Anthocyanins and Related Compounds in Potatoes (*Solanum tuberosum* L.)

Christine Elaine Lewis

A thesis submitted in
partial fulfilment of the
requirements for the degree of
Doctor of Philosophy in Biochemistry
in the Department of Plant and Microbial Sciences
at the University of Canterbury

University of Canterbury (1996)
Christchurch, New Zealand

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

2-D	two-dimensional
4CL	4-coumarate:CoA ligase
A	absorbance
ABA	abscisic acid
AM	amylose
anth-1	petunidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside
anth-2	malvidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside
anth-3	pelargonidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside
AP	amylopectin
ara	arabinoside
BA	benzyladenine
BAW	*n*-butanol : acetic acid : water (4:1:2.2)
BBPW	*n*-butanol : benzene : pyridine : water (5:1:3:3, upper phase)
BSA	bovine serum albumin
C4H	cinnamic acid 4-hydroxylase
CD	cyclodextrin
CHI	chalcone isomerase
CHS	chalcone synthase
cv.	cultivar
Cy	cyanidin
DHR	dihydroflavonol reductase
DG	tissue-cultured plantlets grown in the dark
dOHKm	dihydrokaempferol
dOHMy	dihydromyricetin
dOHQu	dihydroquercetin
Dp	delphinidin
DPO	diphenyloxidase
DT	tissue-cultured minitubers grown in the dark
DTT	dithiothreitol
ER	endoplasmic reticulum
F3H	flavanone 3-hydroxylase
F3'H	flavonoid 3'-hydroxylase
F3',5'H	flavonoid 3',5'-hydroxylase
FAB-MS	fast atom bombardment mass spectroscopy
FS	flavonol synthase
FW	fresh weight

GA	gibberellic acid
gal	galactoside
glu	glucoside
GT	glycosyltransferase
HIR	high irradiance response
HMF	5-hydroxy-methylfurfural
HOAc	acetic acid
HPLC	high performance liquid chromatography
IAA	indole acetic acid
Jcm^{-2}	Joules per centimetre squared
Km	kaempferol
l	length of tuber
LG	tissue-cultured plantlets grown in the light
LT	tissue-cultured minitubers grown in the light
M	moles per litre
MeOH	methanol
mM	millimoles per litre
MS	mass spectroscopy
Mv	malvidin
Mv-U	extract from Urenika tubers - mainly malvidin glycosides
MW	molecular weight
My	myricetin
N1	top node from a tissue-cultured plantlet with a pair of leaves
N2	second node from the top with a pair of leaves
N3	third node from the top with a pair of leaves
NA-reagent	Naturstoffreagenz A reagent
NaOAc	sodium acetate
nmr	nuclear magnetic resonance
PAL	phenylalanine ammonia-lyase
PAL-IS	phenylalanine ammonia-lyase inactivating system
PC	paper chromatography
Pfr	phytochrome (far-red)
Pg	pelargonidin
Pg-RF	extract from Red Flesh tubers - mainly pelargonidin glycosides
PMM	potato multiplication medium
PMSF	phenylmethylsulfonylfluoride
Pn	peonidin
polyD	polydextrose
Pr	phytochrome (red)

Pt	petunidin
PVPP	polyvinylpolypyrrolidone
Qu	quercetin
Qu-gly	quercetin glycoside
RKE	Red King Edward
RP	reversed phase
rut	rutinoside
SA	surface area
SAM	*S*-adenosyl-L-methionine
se	standard error
sh	shoulder
subsp.	subspecies
TAL	tyrosine ammonia-lyase
TFA	trifluoroacetic acid
TG	tangential
TIM	tuber initiation medium
TLC	thin layer chromatography
TV	transverse
w	width of tuber
xyl	xyloside
λ_{max}	wavelength of maximum absorbance
%DM	percentage of dry matter

ABSTRACT

Coloured potatoes may have economic value as natural food colourants and as food products such as novelty potato crisps and coloured potato salads. This thesis investigated the biochemistry and physiology of anthocyanins and related compounds in *Solanum tuberosum* L., and the relationship to tuber colour. These factors were discussed in terms of consumer requirements.

Phenolic acids, flavonoids and anthocyanins were surveyed and quantified in the tubers (skin and flesh), flowers and leaves of twenty nine cultivars of *S. tuberosum* and eight other *Solanum* species (*S. acaule, S. berthaultii, S. gourlayi, S. oplocense, S. sanctae-rosae, S. sparsipilum, S. speggazzinii, S. stenotomum).* The main anthocyanin found in red tubers was pelargonidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside (200-2000μg/gFW) with lower amounts of peonidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside (20-400 μg/gFW). Light to medium purple coloured tubers contained mostly petunidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside (400-2000μg/gFW) plus low concentrations of malvidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside (20-200μg/gFW), whilst dark purple/ black coloured tubers contained a similar concentration of petunidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside (1000-2000μg/gFW) to the light to medium purple tubers, but with high concentrations of malvidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside (2000-5000μg/gFW). Red and purple tubers also contained a number of minor anthocyanins, with the same aglycones as above, but mostly as the 3-rutinosides. Tubers contained high concentrations of phenolic acids (2000-5000μg/gFW), with chlorogenic acid making up 60-90%. Apart from the anthocyanins, there were low concentrations of other flavonoids (200-300μg/gFW). The major anthocyanin present in the flowers was petunidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside. Flowers and leaves contained higher concentrations of flavonoids (1000-3000μg/gFW), the major flavonoids being quercetin-glycosides. The flavonoid patterns of flowers and leaves fell into two different categories with some cultivars containing high concentrations of quercetin-glycosides, whilst others contained low concentrations.

Tubers of the other available *Solanum* species did not show the range of colours shown by *S. tuberosum* cultivars, and were mostly white or light purple, with petunidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside being the major anthocyanin (when present) in the skin of tubers of the other *Solanum* species. The major anthocyanin in flowers of the other *Solanum* species was petunidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside, with levels similar to those found in *S. tuberosum* flowers. Low levels of anthocyanin were found in the leaves of the other *Solanum* species whereas in *S. tuberosum* no

anthocyanins were found in the leaves. There was considerable variation among plants and species in both phenolic acid and flavonoid concentrations, but generally a similar pattern was found in the other *Solanum* species as in *S. tuberosum* cultivars, except that *S. tuberosum* flowers contained lower concentrations of total phenolic acids and flavonoids on average, and tubers and leaves contained high concentrations of flavonoids.

The expression of different pathways appeared to depend on the species, plant tissue and environmental factors. Diseased tubers contained higher concentrations of phenolic acids, flavanones and flavonols than healthy tubers, and some flavonols which were not present in healthy tubers were produced in diseased tubers. There was differential expression of anthocyanins, flavonoids and phenolic acids in the different parts of the plant (tubers, flowers and leaves). These compounds also responded differently to light, with anthocyanins showing a large increase, flavonoids a smaller increase, and phenolic acids no change in concentration, in minitubers after indirect exposure to light.

The biosynthesis of anthocyanins in tubers was investigated throughout tuber development and during storage. Newly initiated tubers contained no anthocyanin, and subsequent production of colour occurred firstly at the stem end of the developing tuber, and then proceeded to the bud end. Anthocyanin concentrations increased throughout the development of the tuber, reaching a maximum at a tuber size which was dependent on the cultivar (about 150-200g for Desirée). Concentrations were higher at the stem end of the tuber than the bud end for most of tuber development although, as the maximum anthocyanin concentration was reached, the distribution of anthocyanin over the tuber became more uniform. This suggested that the transport of some compound (carbohydrate or "trigger") was responsible for the initiation of anthocyanin biosynthesis. Concentration of other flavonoids also increased and followed a similar pattern to that of the anthocyanins, with maximum concentrations occurring in Desirée tubers about 150-200g. Phenolic acid concentrations also increased during tuber development, although these reached a maximum concentration in slightly smaller tubers (about 70-100g).

Microscopical studies of anthocyanin-containing cells showed that the difference in colour intensities of the different cultivars was because of differences in the amount of anthocyanin produced in individual cortex cells, in the proportion of cortical cells within a layer producing anthocyanin, and the number of layers of coloured cells. Totally white tubers did not contain any anthocyanin coloured cells, whilst more highly coloured tubers contained a greater number of more highly coloured cells in these layers. Additionally, the strongly coloured tubers had increased amounts of anthocyanin present in the phellem cells and intensely coloured deposits of anthocyanin around the cell walls of these cells.

Cold storage (4°C) of tubers caused an increased concentration of anthocyanins, especially at the bud end of the tuber, so that the distribution pattern of anthocyanins was reversed from that found in developing tubers. Storage at higher temperatures (10°C and above) caused a decrease in anthocyanin concentration. These changes in anthocyanin concentration were thought to be related to sprouting and also the sugar concentration within the tuber. Cooking (boiling, steaming or crisping) of tubers (until they were ready to eat) did not result in any significant loss in anthocyanin colour, although after two to three times the normal cooking time some loss of anthocyanin colour occurred.

Anthocyanin colour may have been affected by the high concentration of starch or sugars found in tubers. Addition of amylose, amylopectin, α-cyclodextrin and β-cyclodextrin to anthocyanin solutions caused a decrease in anthocyanin colour, whilst the addition of sugars (glucose, sucrose and maltose) resulted in increased anthocyanin colour.

Tissue-cultured minitubers were used to investigate the effects of light on anthocyanin biosynthesis. Although anthocyanins were produced in the dark in field grown tubers, the presence of light on the plant leaves was necessary for anthocyanin production in some cultivars. Anthocyanin concentration increased with increased light intensities in all cultivars studied (to a maximum of about 3.2ng/cm^2 of surface area in Desirée minitubers), and the activities of enzymes (phenylalanine ammonia-lyase, cinnamic acid 4-hydroxylase, chalcone isomerase, flavanone 3-hydroxylase, flavonoid 3'-hydroxylase, dihydroflavonol reductase and glycosyltransferase) showed related increases. Biosynthesis of anthocyanins was a high irradiance response and required at least eight hours of exposure to light for a significant increase in anthocyanin concentration to occur, after which anthocyanin concentration (to a similar maximum concentration) increased linearly with increasing time of exposure. Both phytochrome and cryptochrome light receptors were thought to be involved because light of blue, red and purple wavelengths enhanced anthocyanin production, compared with white light of similar intensity.

The regulation of anthocyanin biosynthesis in potato tubers is different from most other plants and tissues because direct light exposure of the tuber is not necessary for anthocyanin production. However, for maximum anthocyanin synthesis the exposure of the plant leaves to light is required. It is proposed that this synthesis of anthocyanins in tubers in the dark requires genetic capability of the tubers, a supply of carbohydrates, and is mediated by a "trigger" compound produced after the exposure of the leaves to light and transported to the tubers.

CHAPTER 1

Introduction

1.1 The potato

Potatoes (*Solanum tuberosum* L.) are a crop of high nutritive value and of considerable world importance, being fourth in world production, following wheat, maize and rice. The cultivated potato, *S. tuberosum,* belongs to the family Solanaceae, comprising over 2,000 species. There are several poisonous species in this family, but also many food plants of economic importance (Timberlake and Bridle, 1982). Other well known crops in the Solanaceae include the tomato (*Lycopersicon esculentum*), the egg plant (*S. melongena*), various species of chilli peppers (*Capsicum* spp.) and tobacco (*Nicotiana tabacum*). Many other common species also belong in this family; the alkaloid drug plants, such as *Atropa, Hyosyamus, Scopolia, Mandragora,* and Poroporo (*S. aviculare*), the ornamentals in the genera *Petunia, Nicotiana* and *Schizanthus,* as well as a range of tropical fruits such as pepino (*S. muricatum*), lulu or narajilla (*S. quitoense*), tree tomato (*Cyphomandra betacea*) and husk tomato or cape gooseberry (*Physalis* spp.).

There is a total of 235 recognised tuber-bearing *Solanum* species, of which 7 are cultivated and 228 are wild (Hawkes, 1990). The wild species are widely distributed through much of the Americas, from the south-west of the United States of America, into nearly every state in Mexico, and through into Guatemala, Honduras, Costa Rica and Panama. In South America they occur in almost every country, but are found mostly in Venezuela and Argentina, and the Andes of Colombia, Ecuador, Peru, and Bolivia. From a few of these wild species the seven cultivated species of potato have arisen, occurring in a range of chromosome numbers including 24 (diploid), 36 (triploid), 48 (tetraploid) and 60 (pentaploid). Of these cultivated species, *S. tuberosum* is a tetraploid and its subspecies *tuberosum* is the common potato with world-wide distribution. Another subspecies of *S. tuberosum,* subspecies *andigena,* is commonly cultivated in the South American Andes, whilst the other six cultivated potato species are restricted to the high Andes of South America in an area stretching approximately from central Peru to central Bolivia (Hawkes, 1990). Potato systematics will be discussed further in Section 3.1.3.

1.1.1 Cytology - ploidy levels

The presence of polyploidy with a base number of 12 in wild potatoes was established by Smith (1927). Later workers (reviewed in Hawkes, 1990) showed that there was not one, but many, polyploid series in wild potatoes which had evolved independently. Diploids, tetraploids and hexaploids are sexually fertile but the odd numbered polyploids are sterile. Despite the presence of polyploids, 73% of the tuberous *Solanum* spp. are diploid. There are three cultivated diploids (*S. stenotomum, S. phureja* and *S. ajanhuiri*), two triploid species (*S. chaucha* and *S. juzepczukii*), and one tetraploid species *S. tuberosum* with its two subspecies, *tuberosum* and *andigena* (Hawkes, 1990).

1.1.2 The early potato and introduction into Europe

The potato was first discovered during the conquest of Peru in the 1530's when the potato was a widespread crop in the Andes and in Chile. There is no reliable account of the introduction of the potato into Europe, however Hawkes (1990) found that all the evidence pointed to two early introductions. The first was into Spain in about 1570 and the second was into England between 1588 and 1593. These first European potatoes were the short-day adapted subspecies *andigena* cultivars from the Andes, probably Colombia.

1.1.3 Further spread into Europe

The short-day adapted subspecies (*andigena*) was not suited to the European climate, and following selection and plant breeding the long-day adapted *tuberosum* subspecies evolved. The potato was widely grown in Spain and Italy by the late 1600's and in France by the mid 1700's (Brown, 1990). However, there was considerable resistance to their cultivation by all except the poorest people until they were popularised by Parmentier in 1773. Their general adoption in Eastern Europe occurred between the late 1700's to the early 1800's, when the adaptation to short-day length had been bred out and the plants were able to tuberize in the long-day lengths found in most of Europe. The potato was completely unknown in North America until the early 1600's. Potatoes were taken to India and China by British missionaries in the late 1600's and were known in Japan and parts of Africa by about the same period (Hawkes, 1990; Simmonds, 1976). In New Zealand they appeared in 1769 and had been adopted by the Maoris by 1840, who were already conversant with sweet potato cultivation (Yen, 1961/2).

Figure 1.1 Range of colours displayed by potato tubers.

1.2 Introduction to pigments in potato tubers

The appearance of food is important to consumers, and a major factor determining appearance is the colour. Within cultivars of *S. tuberosum*, tubers may display many colours from white, through yellow, brown, pink, red, purple, purple-blue, to almost black (Figure 1.1). There are two main classes of pigments commonly found in potato tubers, the carotenoids and anthocyanins, however after exposure to light a third class of pigment, the chlorophylls, may be present. Because the colour of tubers is so important these classes of pigments are discussed in more detail.

1.2.1 Carotenoids

The flesh of all varieties of potato is tinged with yellow to a greater or lesser extent, and this is due mainly to the presence of carotenoids, a class of plastid pigments. The main carotenoid constituents in potato tubers are the xanthophylls; violaxanthin, lutein and lutein-5,6-epoxide, with small amounts of neoxanthin and neoxanthin-A. β-Carotene, a common carotenoid in many other plants, and also present in the aerial parts of the potato plant, is absent or present only in trace amounts in the tubers (Burton, 1989). There is a direct correlation between yellow flesh colour and total carotenoid content, which is a heritable characteristic. It is thought that the tendency for a high carotenoid content is determined by a single dominant gene, although there are modifying genes (Brown *et al.*, 1993).

1.2.2 Anthocyanins

The other major group of pigments which may occur in the tubers of some potato cultivars is the anthocyanins. These are vacuolar pigments which give rise to the red to purple/black colourations in the tuber skin and/or flesh (Figure 1.1), and these colours often mask the colours produced by carotenoids. Anthocyanins belong to a class of compounds called the flavonoids, however most other flavonoids are usually colourless or have a yellow colour. Other flavonoids, such as flavonols and flavones which have a yellow colour, may be present in trace amounts, although it is unlikely that they have any significant effect on flesh or skin colour (Burton, 1989).

1.2.2.1 Classification of anthocyanin colour in potato plants

The colour and distribution of anthocyanin pigmentation over various parts of the plant are characteristics which are of great value in the identification of a cultivar. The tuber skin may be wholly or partially coloured. In partially coloured tubers the pigmentation

may be confined to the eyes or eyebrows only, or may be splashed, when it is confined to areas around the eyes. Alternatively, when areas around the eyes have no pigmentation and the remainder of the tuber is pigmented, the distribution is described as spectacled. If pigmentation is distributed at random in one or more areas around the tuber it is scattered, and if the surface is more or less uniformly covered with pigmented spots it is stippled (Burton, 1989). The flesh of the tuber may also be classified by the various distributions of anthocyanin which occur. The colour of sprouts (sprouted under natural diffused light) also varies between cultivars (Burton, 1989; Sparks, 1990). The distribution and colour of anthocyanin pigmentation over other parts of the potato plant are also useful in the identification of potato cultivars. For example; the anthocyanin colour is classed as either present or absent in the stem, flower peduncle, flower pedicel, and flower bud. The colour of the upper and lower surface of the flowers is a distinctive characteristic of different potato cultivars and is categorised into four groups: white, light purple, deep purple and blue. Also distinctive is the flower colour distribution, with coloured flowers often white tipped due to the absence of anthocyanin colouration in the petal tips (Sparks, 1990).

1.2.3 Other pigments

Greening, due to chlorophyll formation as a result of exposure to light, is not uncommon but, this is usually accompanied by the production of the toxic glycoalkaloids such as solanine and chaconine (Burton, 1989), and therefore tubers showing the green colouration should not be eaten. Melanin formation as a result of injury may give rise to grey or black discolourations, which are also undesirable.

Thus, the major contribution to colour of potato tubers is from the anthocyanins and this thesis will focus on the biochemistry and physiology of these and related compounds.

1.3 Biochemistry and chemical structures of the phenolics

Whilst the main aim of this project was to study the coloured anthocyanin pigments in potatoes, it was also considered necessary to include a study of compounds which were closely related both structurally and biosynthetically, to enable an insight into the biochemistry of the anthocyanins.

The anthocyanins and flavonoids belong to a wider class of compounds called phenolics. The term "phenolic" is defined chemically as a substance which contains an aromatic ring with a hydroxyl substituent (including esters, methyl ethers, glycosides, etc.) (Harborne,

1989), although most plant phenolics have two or more hydroxyl groups. However, a purely chemical definition of a plant phenolic is not entirely satisfactory because it would mean including some compounds which are principally terpenoid in origin, so a biogenetic definition is preferable. The natural plant phenolics arise biogenetically from two main pathways; the shikimic acid pathway and the acetate (polyketide) pathway, and these are discussed further in Chapter 5.

1.3.1 Flavonoids

As well as the anthocyanins, the flavonoid class of compounds includes the flavonols, flavanones, flavones, catechins, aurones, and chalcones. These flavonoid classes are closely related chemically and all have a basic fifteen carbon nucleus with a $C_6C_3C_6$ structure composed of two aromatic rings (A and B), linked by a three carbon chain (Figure 1.2). This chain is closed in most flavonoids to form the heterocyclic C-ring, but remains open in the chalcones and dihydrochalcones.

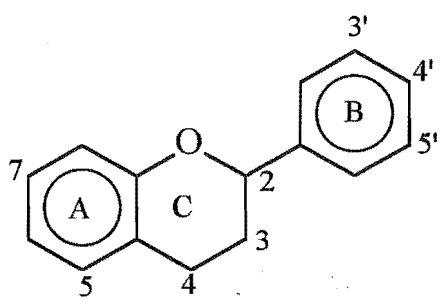


Figure 1.2 Generalised structure and numbering system of carbon atoms of most flavonoids.

Flavonoids are abundant in nature and are found in all green plants except algae and hornworts, and occur in virtually all plant parts including roots, stems, leaves, bark, flowers, pollen, nectar, berries and seeds (Harborne, 1967; Markham, 1982). There is a general trend towards a wider range of flavonoids being present in more highly evolved plant groups, and taxonomically related plants are generally found to produce similar types of flavonoids with similar hydroxylation, methylation, glycosylation and acylation patterns (Harborne, 1972). Diversity and complexity of the flavonoids depend on the variety of the aglycones and the high number of glycosylation and acylation patterns, and also their association into complex molecules (Gross, 1987).

It has been estimated that about 1.5% of the carbon fixed annually in photosynthesis is used for the synthesis of flavonoids (Smith, 1972). The function of flavonoids in plants is not completely understood but flower colours, together with scent and nectar, attract insects to plants and ensure cross fertilisation. Some leaf flavonoids may be involved in insect feeding responses as specific deterrents, and flavonoids can act as a screen against damaging UV radiation (Harborne, 1976), and also as protective agents against infection by phytopathogenic organisms (Hahlbrock, 1981). Until recently flavonoids were only recorded as vacuolar constituents or in leaf waxes and bud exudates, but Strack *et al.* (1989) have reported that they can also be linked to the cell wall.

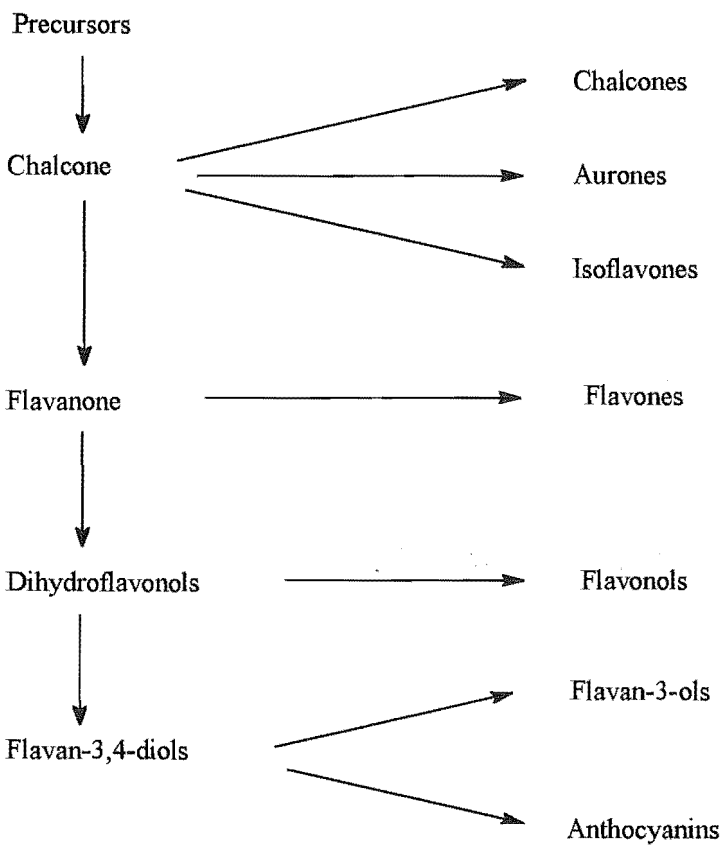


Figure 1.3 Biosynthetic pathway for flavonoid classes.

In plants, flavonoids occur in a variety of structural forms, but the early steps in their biosynthesis are closely interrelated. Early experiments with radioactively labelled precursors established that the carbon skeleton of all flavonoids is derived from acetate and phenylalanine (Hahlbrock, 1981). A central intermediate in the formation of all flavonoids is the chalcone (Hahlbrock 1981) and all other classes are derived from this by a variety of routes (Figure 1.3). The basic $C_6C_3C_6$ structure is formed from the condensation of 4-coumaroyl CoA, two malonyl CoA's and acetyl CoA, a reaction

catalysed by the enzyme chalcone synthase (CHS) (Mancinelli, 1985). The reactions leading to the formation of 4-coumaroyl CoA form the general phenylpropanoid pathway and are common to the pathways of the biosynthesis of a number of classes of compounds, including flavonoids, phenolic acids, cinnamate esters and lignins (Mancinelli, 1985). The A-ring is formed from three acetate units (from malonyl CoA), whilst phenylalanine (from the shikimic acid pathway) gives rise to the B-ring and carbons 2, 3 and 4 of the heterocyclic C-ring (Figure 1.2). The biosynthesis of flavonoids is discussed further in Chapter 5.

The flavonoids are divided into classes depending on the oxidation state of the C-ring (Figure 1.4). Individual carbon atoms are identified by a numbering system that uses ordinary numerals for the A- and C-rings and "primed" numerals for the B-ring (Figure 1.2 and 1.4). However, due to structural changes, a modified numbering system is used for chalcones and aurones.

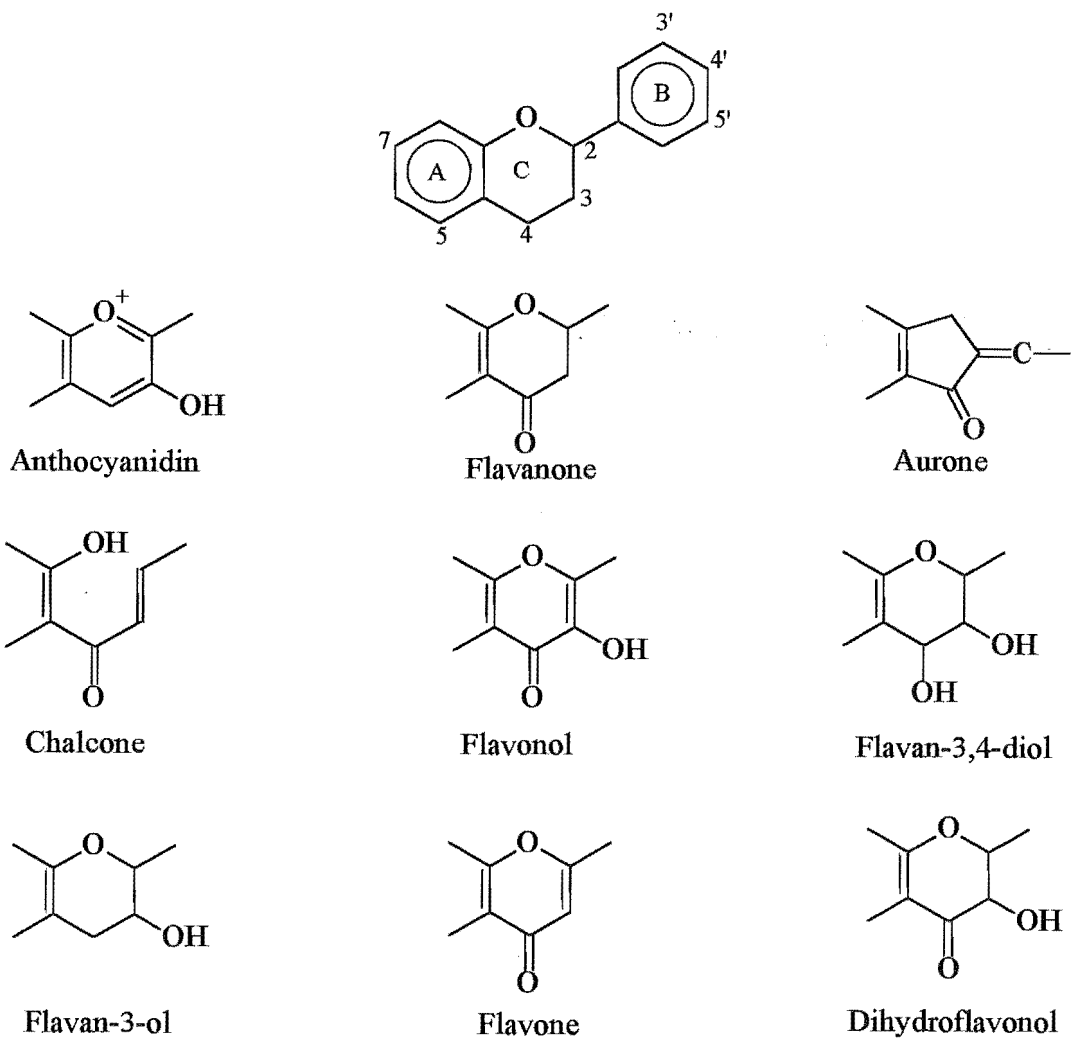


Figure 1.4 The C-ring structure of the main classes of flavonoids.

Within each class of flavonoid (e.g. anthocyanins, flavonols, flavanones, flavones etc.) the basic fifteen carbon skeleton is converted into the many different compounds by hydroxylation of the A- and B-rings, methylation and glycosylation of these hydroxyl groups, and sometimes acylation. These reactions are common to all classes of flavonoids, and therefore will be discussed before a more detailed discussion of the different classes of flavonoids.

1.3.1.1 Hydroxylation

A number of hydroxylation patterns are observed among the flavonoids, but some patterns occur more frequently than others. The most common hydroxylation pattern of the A-ring is the 5,7-hydroxylation pattern but occasionally 5,7,8- or 5,6,7-hydroxylation patterns are found. The B-ring generally has a 4'-, 3',4'- or 3',4',5'-hydroxylation pattern, but there are some rare flavonoids which lack B-ring hydroxylation (Stafford, 1990).

1.3.1.2 Glycosylation

Plant flavonoids are usually glycosylated. This makes the flavonoid more stable and water soluble which is necessary for the retention of some flavonoids in the aqueous medium of the vacuole. There are two major types of glycosidic linkages found, forming *O*-glycosides or *C*-glycosides (Stafford, 1990), but *O*-glycosides are more common and only these have been found in potatoes. Flavonoid *O*-glycosides are formed by an acid-labile acetal bond between a sugar and a flavonoid hydroxyl group (Markham, 1982), and although any of the hydroxyl groups on the flavonoid nucleus may be glycosylated, some have a higher probability of being so than others. Thus, flavones, isoflavones and dihydroflavones are usually glycosylated at the 7-hydroxyl, whilst in flavonols and dihydroflavonols the 3-hydroxyl is usually glycosylated, with sometimes the 7-hydroxyl as well. Anthocyanins almost always have a glycosyl group attached at 3-hydroxyl. When more than one sugar is present in the anthocyanin the other sugar(s) can be attached to one of the other hydroxyls, and 5-, 7-, 3'-, 5'- or even 4'-glycosides may be formed (Ebel and Hahlbrock, 1982).

Glucose is the most common sugar to be attached, but galactose, rhamnose, xylose and arabinose are also relatively common, with other sugars detected occasionally. These sugars are all usually found in the pyranose form (except for arabinose, which may occur in both furanose and pyranose forms). The *D*-sugars (glucose, galactose and xylose) are usually β-linked to the anthocyanidin, whilst the *L*-sugars (arabinose and rhamnose) are α-linked (Harborne, 1967). Di- and tri-saccharides may also be linked to the flavonoids,

for example, rutinose (*O*-α-L-rhamnosyl-D-glucose) and sophorotriose (*O*-β-D-glucose-*O*-β-D-glucosyl-D-glucose).

1.3.1.3 Methylation

Methylation of the hydroxyl groups of anthocyanins is believed to occur after the fifteen carbon skeleton has been formed (see Figure 1.4), although methylation of cinnamic acid derivatives can occur at the phenylpropanoid level (e.g. ferulic and sinapic acids), and feruloyl-CoA can serve as a weak substrate for chalcone synthase in some cases (Hahlbrock and Grisebach, 1975). Methylation commonly occurs after the glycosylation and even acylation steps, usually occurring at the 3'- and 5'-positions and less frequently at positions 5 and 7 (Stafford, 1990).

1.3.1.4 Acylation

Acylation with aromatic hydroxy-cinnamic acids, such as caffeic, *p*-coumaric, and ferulic acids, and with aliphatic acids, such as malonic acid, via acyl transferases is common in many flavonoid classes. These acyl groups are usually attached to sugars. Malonylation is considered to be important for vacuolar transport of anthocyanins in cell cultures of *Daucus carota*, because malonated anthocyanins are more stable and also more polar which assists in their transport into the aqueous vacuole (Stafford, 1990).

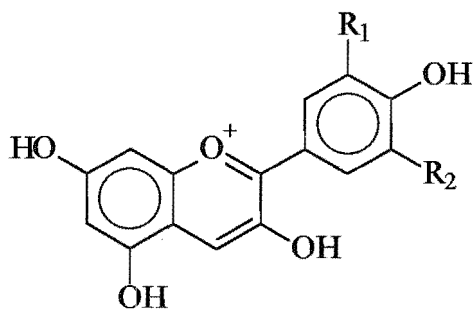
Because of the complexity of the flavonoid classes, the biochemical properties and structures of compounds in the various classes of flavonoids will be reviewed.

1.3.1.5 Anthocyanins

The basic anthocyanidin (aglycone) structure is given in Figure 1.5. Anthocyanins (glycosylated anthocyanidins) are typically pigments of fruits, flowers and leaves and, whilst they do occur in other parts of the plant, they are often confined to, or occur in highest concentration in, one kind of tissue. Deeply coloured flowers may be produced on plants with essentially anthocyanin free stems and leaves however, in general, the capacity of a plant to synthesise anthocyanin at all results in the formation of at least traces of the pigment in the green parts of the plant (Geissman, 1962).

Anthocyanins are water soluble, vacuolar pigments responsible for the violet, purple, blue, red and pink colours of stems, leaves, flowers, fruits and roots in the vast majority of higher plants (Mancinelli, 1985). Six anthocyanidins are widely distributed in plants:-

pelargonidin (Pg), cyanidin (Cy), delphinidin (Dp), peonidin (Pn), petunidin (Pt) and malvidin (Mv) (Figure 1.5). Anthocyanidins other than these are few in number and are very rare in occurrence (Harborne, 1967). All six of these anthocyanidins have been found in *Solanum* species (Harborne, 1960a; 1967). The colour of anthocyanins in the plant is determined by the number and position of hydroxyl and methyl groups on the B-ring (*i.e.* the identity of the aglycone), the nature and number of sugars attached to the anthocyanidin and the position of the attachment, the nature and number of the aliphatic or aromatic acids attached to the sugar, and the physiochemical medium in which they are viewed (Mazza and Brouillard, 1990) (see Section 2.1 for further discussion). Anthocyanins are usually glycosylated at the 3-hydroxyl to provide stability (see Section 2.1.1.1), and the most common anthocyanin glycosides include the 3-glucoside, 3-galactoside, 3-rutinoside and 3-sophoroside (Harborne, 1967).



$R_1 = R_2 = H$; pelargonidin

$R_1 = OH$, $R_2 = H$; cyanidin

$R_1 = R_2 = OH$; delphinidin

$R_1 = OCH_3$, $R_2 = H$; peonidin

$R_1 = OH$, $R_2 = OCH_3$; petunidin

$R_1 = R_2 = OCH_3$; malvidin

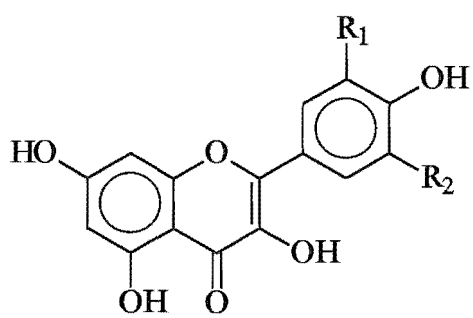
Figure 1.5 Structures of the common anthocyanidins.

1.3.1.6 Flavonols

Flavonols are the most variable and most abundant of all the flavonoid classes (Stafford, 1990). The flavonol and anthocyanin classes are closely related biosynthetically, and these compounds are frequently found together in the plant cell (Hahlbrock, 1981). Flavonols are more highly oxidised and more stable than anthocyanins, and have also been found in *Solanum* species (Wietschel and Reznik, 1980a and b). From the point of view of colour, anthocyanins are usually more important, but in some plants flavonols

may contribute to the colour, either as yellow pigments in their own right, or as copigments with anthocyanins (Harborne, 1967) (discussed in Section 2.1). Flavonols are usually found in the plant as glycosides in the cell vacuole, but aglycones may also be found excreted as exudates or attached to the cell wall (Strack *et al.*, 1989).

Kaempferol (Km) and quercetin (Qu) are the most common flavonols, with the aglycones or glycosides of these being found in 50% of plant species, whilst myricetin (My) and My-glycosides are present in 10% of plants (Figure 1.6) (Harborne, 1967). Isorhamnetin is found in some plants and is formed by the methylation of the 3'-hydroxyl group of the B-ring (Figure 1.6). All four of these flavonols are commonly found in the flowers and leaves of plants, and have been found in the tuber-bearing *Solanum* species (Wietschel and Reznik, 1980a and b). Flavonol glycosides often have similar glycosylation patterns to the anthocyanins within a plant species, and are usually highly glycosylated.



$R_1 = R_2 = H$; kaempferol

$R_1 = OH$, $R_2 = H$; quercetin

$R_1 = R_2 = OH$; myricetin

$R_1 = OCH_3$, $R_2 = H$; isorhamnetin

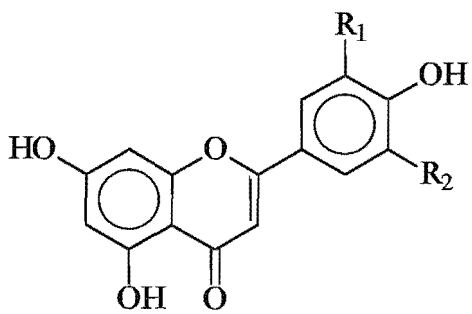
Figure 1.6 Structure of common flavonols.

1.3.1.7 Flavones

Flavones are the second most variable and abundant of all the flavonoid classes (after flavonols). Flavones (Figure 1.7) differ from flavonols (Figure 1.6) only by the absence of a hydroxyl group at the 3-position in flavones, however the two groups differ in their spectral and colour properties, and can be distinguished by chromatographic procedures. These two groups have very similar biosynthetic pathways, with several steps in the biosynthesis catalysed by the same enzymes. The glycosylation patterns in flavones

differs from that in anthocyanins and flavonols, because in anthocyanins and flavonols the most common glycosylation occurs at carbon-3. This glycosylation does not occur in the flavones because there is no hydroxyl group at the 3-position. The most common flavone glycosides have a sugar (usually glucose) at the 7-position, but a number of 5-glycosides are known (Ribéreau-Gayon, 1972). Flavones have a yellow colour and may act as copigments with anthocyanins (Harborne, 1967). They are found in the plant as glycosides in the cell vacuole, or as aglycones usually outside the cell (e.g. in exudates).

The flavones, apigenin and luteolin (Figure 1.7), are widely distributed in the angiosperms, and have also been found in *Solanum* species (Harborne, 1967; Stafford, 1990). Another flavone, tricin, is common only in grasses.



$R_1 = R_2 = H$; apigenin

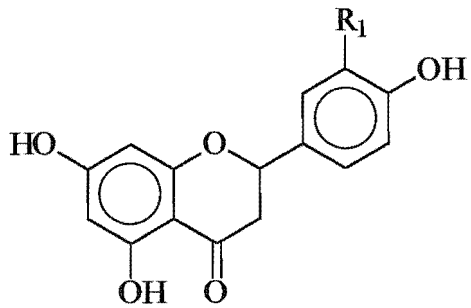
$R_1 = OH$, $R_2 = H$; luteolin

$R_1 = R_2 = OCH_3$; tricin

Figure 1.7 Structure of common flavones.

1.3.1.8 Flavanones

The flavanones are colourless flavonoids, similar in structure to the flavones, except they contain a single bond in the C-ring (Figure 1.8), instead of the double bond found in the flavones (Figure 1.7). Naringenin and eriodictyol (Figure 1.8) are the best known flavanones, and are fairly widely distributed. Each flavanone is capable of existing in two optical isomeric forms because of the asymmetric carbon at position 2. They are usually found as free aglycones (un-glycosylated) (Stafford, 1990).



R_1 = H; naringenin

R_1 = OH; eriodictyol

Figure 1.8 Structure of common flavanones.

1.3.1.9 Proanthocyanidins and leucoanthocyanidins

Another important group of flavonoids is the proanthocyanidins. The term proanthocyanidin includes all compounds that produce anthocyanidins by cleavage of a carbon-carbon bond when heated with acid (Porter, 1988). Included in this group are the commonly occurring flavan-3-ols, of which the diastereoisomeric pair (+)-catechin and (-)-epicatechin are frequently found in plants (Figure 1.9). Proanthocyanidins also include dimers and trimers composed of catechin, epicatechin and/or anthocyanidin units. Proanthocyanidins accumulate mainly in the cell vacuoles, although they are also found to be associated with lignin in the cell walls of some species (Swain, 1979; Stafford, 1988). These flavonoids are usually found as aglycones, rather than glycosylated, which is probably a reflection of their greater solubility in aqueous media than most other flavonoids (Stafford, 1990), however some substitution such as glycosylation, methylation and esterification has been found (Stafford, 1993).

There is some confusion in the literature with the terms "leucoanthocyanidin" and "proanthocyanidin", and to which compounds are being referred. The definition of leucoanthocyanidins that will be used here includes all monomeric flavonoids that produce anthocyanidins by cleavage of a carbon-oxygen bond on heating with a mineral acid (Porter, 1988). Leucoanthocyanidins, including flavan-4-ols and flavan-3,4-diols (precursors of anthocyanins in the biosynthetic pathway), are frequently found in the wood and bark of trees.

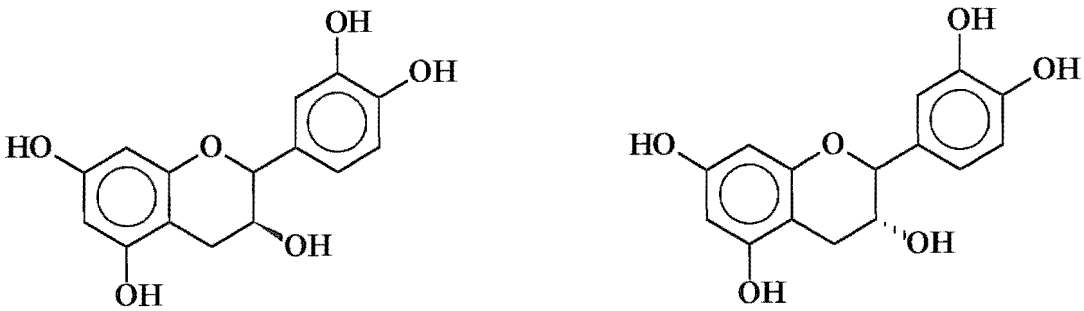


Figure 1.9 Structure of common flavan-3-ols a) catechin and b) epicatechin.

1.3.1.10 Other flavonoids

Other flavonoids include the pale yellow coloured flavanonols (2,3-dihydroflavanols) (e.g. dihydroquercetin (taxifolin) which is important in the biosynthesis of anthocyanins), the isoflavones which are isomers of the flavones with the B-ring attached to the carbon atom at the 3-position instead of the 2-position, and the strongly yellow coloured chalcone and aurone pigments.

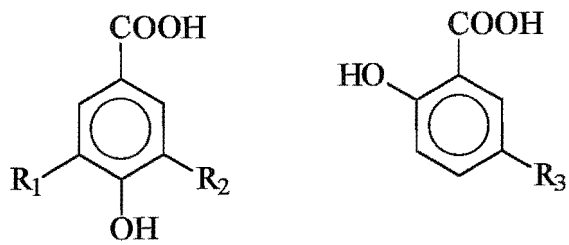
1.3.2 Phenolic acids

The cinnamic acid and *p*-coumaric acid precursors of flavonoid biosynthesis are also phenolics and maybe termed "phenolic acids". According to Gross (1981) the term "phenolic acid" is applicable to a large variety of compounds containing at least one phenolic hydroxyl group and one carboxyl group. It is, however, common practice to use the "phenolic acid" terminology for only a limited number of compounds, such as benzoic and cinnamic acid derivatives (van Sumere, 1989). This thesis included some study of these phenolic acids because cinnamic acid and *p*-coumaric acid represent a branch point in the biosynthesis of a number of compounds, including the flavonoids, anthocyanins, cinnamic acids and benzoic acids.

1.3.2.1 Benzoic acids

The benzoic acids are widely distributed in both angiosperms and gymnosperms, and include derivatives of a basic C_6-C_1 structure (Figure 1.10). *p*-Hydroxybenzoic, vanillic and syringic acids occur in large amounts as constituents of lignin, from which they may

be liberated by alkaline hydrolysis (Walker, 1975). Gallic acid is often found as its dimer ellagic acid, and both are often found associated with polymeric proanthocyanidins in the cell wall. A number of benzoic acids are found in potatoes, and these are discussed in Chapter 3.



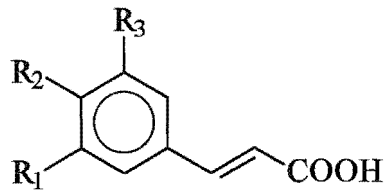
$R_1 = R_2 = H$; *p*-hydroxybenzoic acid
$R_1 = OH, R_2 = H$; protocatechuic acid
$R_1 = OCH_3, R_2 = H$; vanillic acid
$R_1 = R_2 = OH$; gallic acid
$R_1 = R_2 = OCH_3$; syringic acid

$R_3 = H$; salicylic acid (*o*-hydroxybenzoic acid)
$R_3 = OH$; gentisic acid

Figure 1.10 Structure of common benzoic acids.

1.3.2.2 Cinnamic acids

The cinnamic acids are a class of phenolic acids which are derived from a C_6-C_3 structure (Figure 1.11). Four cinnamic acids (*p*-coumaric, caffeic, ferulic and sinapic acids) are widely distributed in plants with *p*-coumaric and caffeic acids by far the most common. Cinnamic acid itself (as opposed to the class of cinnamic acids!!) is also present in some plants but is less common. Because cinnamic acids possess a double bond in the C_3 side chain, they are capable of existing as both *cis* and *trans* isomers. The *trans* isomers are more stable and are the naturally occurring cinnamic acids, however these two isomers are easily interconvertable under the action of light and an equilibrium is reached. Cinnamic acids are sometimes found as glycosides and often occur in conjugated or esterified forms (Ribéreau-Gayon, 1972). A number of cinnamic acids are found in potatoes, and these are discussed in Chapter 3.



$R_1 = R_2 = R_3 = H$; cinnamic acid

$R_1 = H$, $R_2 = OH$, $R_3 = H$; *p*-coumaric acid

$R_1 = R_2 = OH$, $R_3 = H$; caffeic acid

$R_1 = OCH_3$, $R_2 = OH$, $R_3 = H$; ferulic acid

$R_1 = OCH_3$, $R_2 = OH$, $R_3 = OCH_3$; sinapic acid

Figure 1.11 Structure of common cinnamic acids.

1.3.2.3 Cinnamic acid derivatives

There are a number of naturally occurring derivatives of the cinnamic acids. The most common of these is chlorogenic acid (Figure 1.12), which is an ester of caffeic acid and quinic acid (3-caffeoylquinic acid). Chlorogenic acid is one of the major phenolics found in potatoes. Isochlorogenic acid (a mixture of three isomeric dicaffeoylquinic acids) and neochlorogenic acid (5-caffeoylquinic acid) are also found in plants (Ribéreau-Gayon, 1972). Acylated anthocyanins constitute another combined form of the cinnamic acids, where *p*-coumaric, caffeic or ferulic acid are often found linked to a glycosidic hydroxyl of an anthocyanin molecule.

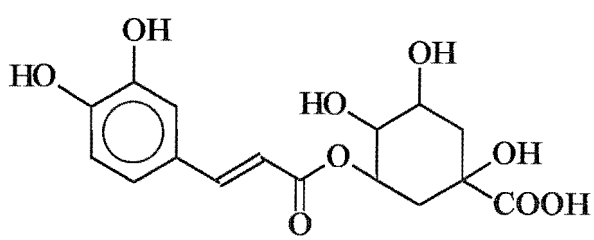
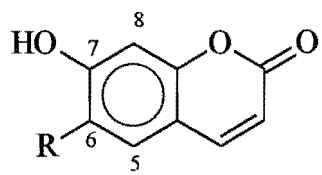


Figure 1.12 Structure of chlorogenic acid.

1.3.3 Coumarins

The other major class of phenolic compounds which is commonly found in plants is the coumarins. The different coumarins are derived from the cyclisation of *o*-hydroxy-*cis*-cinnamic acids. The most common are umbelliferone, aesculetin and scopoletin, whose substitution patterns correspond, to *p*-coumaric, caffeic and ferulic acids respectively (Figure 1.13). Coumarins occur naturally in plants as glycosides, and scopolin (scopoletin-7-glucoside), aesculin (aesculetin-6-glucoside) and cichoriin (aesculetin-7-glucoside) have been found in potatoes (Baruah and Swain, 1959; Harborne, 1960b).



R = H; umbelliferone

R = OH; aesculetin

R = OCH_3; scopoletin

Figure 1.13 Structure of common coumarins.

1.4 Biological importance of phenolics

The phenolics are secondary metabolites, and therefore not required for primary metabolism. So why do plants contain anthocyanins, flavonoids and phenolic acids? What is their role in the biology of the plant? Since one of the aims of this thesis is to understand the biochemistry of flavonoids, and in particular anthocyanins, with a view to the possible alteration in production or stability (*i.e.* change in colour), it is important to understand the answers to these questions, and consider the functions of a particular compound and what effect, if any, the alteration may have on the plant. Therefore, in the following section the biological significance of these classes of phenolics is briefly discussed.

1.4.1 Flavonoids

Probably the most important function of the flavonoids is in the pigmentation of plants, to produce the yellow, red and purple colourations found. Mammals have their highest visual sensitivity around 500nm, the visible λ_{max} of anthocyanins, however a number of insects have visual systems which allow them to view much shorter wavelengths, into the UV range, where flavone and flavonol glycosides have a high absorbance. Just as humans do, a number of other creatures also select their food mainly by the means of visual cues, of which colour plays an important part. The major pollination types (e.g. bee, insect and bird pollination) in flowers can be attributed to the flower colour, and therefore the identity of the flavonoids that are present. Bees generally prefer blue and yellow, butterflies pink and white, moths white, and birds red coloured flowers (McClure, 1975).

Flavonoids (especially flavones and flavonols) absorb strongly in both the UV-A and UV-B regions of the spectrum and are partially responsible for UV protection in plants. This is supported by the following observations about these compounds;

1) they absorb efficiently in the UV range,
2) they are localised preferentially in the epidermis,
3) they accumulate rapidly following UV irradiation and,
4) the most efficient wavelength for induction (290-300nm) is also the most damaging (Jorgensen, 1994).

Flavonoid glycosides are naturally occurring antioxidants in plants. For example, they can serve as antioxidants for ascorbic acid (McClure, 1975), and this flavonoid antioxidant property has been found to be associated with tolerance of ozone and manganese toxicity (both oxidative stresses) in soybean (Foy *et al.*, 1995). Flavonols are used by plants in chemical defence against pathogens and as feeding deterrents to insects and other herbivorous animals. They may interact with proteins and/or cause the inhibition or deactivation of enzymes such as pectinases, plant amylase, phenoloxidase, succinate dehydrogenase, and pancreatic RNAase (van Sumere, 1989). Alternatively they may interfere with the biological processes in cells which can make them toxic to certain micro-organisms or animals, inhibit their growth, or give unpleasant sensations to the taste buds of animals (Grayer, 1989). For instance, Malterud *et al.* (1985) found that flavonoids isolated from the wood of *Salix caprea* were active against some wood-destroying fungi, whilst Weidenbörner and Jha (1993) found that three flavones and one flavanone showed anti-fungal activity against different fungi occurring on various grains. Flavonoids have also been found to promote or inhibit plant growth hormones, for example Qu-glycosides were inhibitors of indole acetic acid (IAA) oxidase whilst Km-

glycosides were cofactors (Stafford, 1990). Another function of some flavonoids is that they act as transcriptional signals from plants to bacteria; for example, certain flavonoids from legumes induce transcription of nodulation (*nod*) genes in N_2-fixing rhizobia bacteria (Phillips, 1992), and acetosyringone (a substituted benzoic acid) induces transcription of virulence (*vir*) genes in *Agrobacterium tumefaciens,* a common plant pathogen (Stachel *et al.*, 1985; 1986).

1.4.2 Phenolic acids

Phenolic acids have also been attributed with divergent physiological and biochemical activities, such as feeding deterrents, defence substances, allelochemicals, chelation of metals to reduce toxicity, plus roles in the inhibition of seed and fungal spore germination, and in plant growth generally. Ferulic acid and coumarin have roles in regulating the germination of barley seeds (van Sumere *et al.*, 1972). Ellagic acid and gallic acid esters are involved as feeding deterrents, defence substances and allelochemicals. Salicylic acid was found to enhance adventitious root formation in mung bean shoots (Kling and Meyer, 1983), and benzoic acids may be of importance in chloroplasts since they have been found to inhibit photosynthesis in spinach chloroplasts (Tissut *et al.*, 1980).

1.5 Economic importance of phenolics

1.5.1 Flavonoids

Flavonoids, including anthocyanins, appear to be non-toxic to man, and have a variety of medical applications. Various flavonoids have been reported to have antibiotic, anti-viral or anti-inflammatory activity (Nakagami *et al.*, 1995; Read, 1995), and a variety of flavonoids have even been found to selectively inhibit HIV infection (Mahmood *et al.*, 1993). Their antioxidant characteristics have a number of uses in medicine, including the scavenging of superoxide anions (Larson, 1995), singlet oxygen (Husain *et al.*, 1987), and lipid peroxy-radicals (Sorota *et al.*, 1982; Castelluccio *et al.*, 1995), as well as the binding of metal ions (Takahama, 1985). Additionally, flavonoids inhibit the oxidation of low-density lipoproteins and reduce the risk of coronary heart disease (Hertog *et al.*, 1993; 1995), and have also been found to have anti-carcinogenic activity (McClure, 1975; Middleton and Kandaswami, 1994). Therefore, the presence of flavonoids with these properties in potatoes are beneficial in such an important food source. Anthocyanins are important economically as natural food colourants, and this is discussed further in Section 1.6.

1.5.2 Phenolic acids

Phenolic acids and their derivatives are used in a number of medical and industrial applications; for example, salicylic acid is the starting material for aspirin and methysalicylate manufacture, gallic acid is an inhibitor of antibody production and clone formation, whereas ellagic acid is a potent inhibitor of the mutagenicity and cytotoxicity activities of benzo(a)pyrene (van Sumere, 1989). Examples of phenolic acids frequently used in industry include, vanillic acid for the manufacture of perfumes, flavourings and pharmaceuticals, and gallic acid which is used in the manufacture of inks and dyes.

1.6 Properties of phenolics in foods

Some flavanones and dihydrochalcones have a distinctive taste, being bitter, sweet, or bittersweet; for instance, certain flavanone glycosides occurring in the peel of *Citrus* fruits are bitter (Grayer, 1989). Studies (reviewed in Harborne, 1967) have found that very small changes in the structure of the flavonoid, such as the position or nature of sugar and other substituents of the A- and B-rings, may cause huge changes in their sensory perception (e.g. taste).

One of the most obvious changes observed when certain fruits and vegetables are cut or damaged is the rapid browning of the exposed surfaces. This enzymatic browning is caused from the oxidation of caffeic acid and its derivatives (compounds containing *o*-dihydroxyl groups) by *o*-diphenyl oxidase (*o*-DPO). Although this phenomenon is generally undesirable, there are a number of instances where it is desirable, for example tea, cocoa and cider all owe their desirable characteristics to oxidised phenolic compounds (Swain, 1962).

Anthocyanins are one of the most important sources of natural food colourants and are of great economic importance. Because they are natural they have become especially important in recent years with the world wide concern over the harmful effects of synthetic food additives. Anthocyanins have many advantages as food colourants; they are naturally occurring, non-toxic and have a wide variety of colours. However, they also possess some serious disadvantages which include the fact that anthocyanins are chemically unstable, their colour is easily affected by a number of reactions occurring in food products, and anthocyanin preparations give about a hundred times weaker colour magnitudes than do synthetic colourants (Mazza and Brouillard, 1987). Thus, anthocyanin colour is affected by factors including, temperature, pH, light, metals,

ascorbic acid, enzymes, sulphur dioxide and copigments (Francis, 1993); some of these factors will be discussed later in Chapter 2.

1.7 Anthocyanin inheritance and genetics in the potato

The production of anthocyanins and other flavonoids is genetically controlled, but only the inheritance and genetics of the anthocyanins have been studied in any detail. It is important to understand the genetical aspects of any compound within a species, as well as the biochemical aspects, particularly if any genetic manipulation to alter its production is envisaged.

The commercially grown potato is a tetraploid, however most of the work carried out into the genetics of *Solanum* species has been carried out using diploid species because they are easier to work with. Therefore, the genetics of both diploid and tetraploid cultivated species are discussed here.

1.7.1 Diploid cultivated species

Early studies on the inheritance of anthocyanins in diploid species was carried out by Dodds and Long (1955) and Harborne (1960a), who carried out thorough studies including the identification of pigments from a number of genotypes. Three independent loci, designated by *P*, *R* and *Ac*, were shown to be concerned with the type of anthocyanin produced in diploid cultivated potatoes. The gene *P* is responsible for the production of purple pigments and controls the production of Dp in both flowers and tubers, and when the *Ac* gene is also present, there is methylation of the Dp to Pt and Mv. As well as methylation, the gene *Ac* is associated with two other effects, the acylation of the anthocyanin molecule with *p*-coumaric acid and the attachment of a glucose residue in the 5-position (Howard, 1969). The gene *R* is responsible for the production of red pigments and controls the synthesis of Cy in the flowers and Pg in the tubers. There is also another allele at the *R* locus, which has been designated R^{pw}, and controls the production of Pn.

The distribution of anthocyanins in the various parts of the plant is determined by several genes. Thus, in order for a specific plant part to be pigmented, a dominant allele of either one, or both of the pigment production genes (*P* and *R*) must be present in addition to a dominant allele of the respective distribution gene for that part of the plant (de Jong, 1987). Dodds and Long (1956) found three genes *B*, *I* and *F* to be closely linked and associated with anthocyanin distribution. The gene *I* controlled distribution of

pigment to the skin of the tubers, gene *F* controlled pigment distribution to the flower, and gene *B* consisted of a series of alleles (B^a-B^d) which controlled the distribution of pigment to the floral abscission layer, the eyebrow on the tuber, a band of pigment on the node of the hypocotyl of the embryo in the seed, and a band of pigment at each node (Dodds and Long, 1956; Howard, 1960). De Jong (1987) proposed an additional gene *Pf* (pigmented flesh) which controls the distribution of anthocyanins to the tuber flesh. Gene *I* was necessary for the expression of the *Pf* locus. A number of other genes have also been described which control the distribution to other parts of the plant (de Jong, 1991).

Besides the colour of the tuber, shape is an important tuber characteristic, especially for food processors, and de Jong and Rowe (1972) found a linkage between genes for tuber shape and skin pigmentation in diploid potatoes.

1.7.2 Tetraploid cultivated potatoes

There are a number of early studies on the inheritance of colour in tetraploid potatoes, mainly *S. tuberosum* (Salaman, 1910; Sirks, 1929; Black, 1933). However, there was a certain amount of confusion because there was no accepted system for the nomenclature and, when reviewing the literature, Swaminathan and Howard (1953) presented a table with 17 different hypotheses for genes involved in tuber colour. Tetraploids are found to have much more complex genetic ratios and therefore are harder to work with than diploids. Howard (1969) considered that major differences were present in anthocyanin pigmentation systems between cultivated tetraploids and diploids, however de Jong (1991), in an analysis of the recent literature, considered that these differences may be due more to differences in the presence or absence of different genes between cultivated tetraploids and diploids, and also to the differences in nomenclature of the genes.

In cultivated tetraploids, the genes *P* and *R* are thought to control similar functions as in diploids (Howard, 1969; de Jong, 1991). No gene similar to the *Ac* gene in diploids has been characterised in tetraploid potatoes but, since most of the anthocyanins present in tetraploids are acylated, a similar gene must exist. As in diploids, there are a number of genes which control the distribution of colour in plant parts. In *S. tuberosum* there is a basic gene, *D*, which is necessary for the development of pigmentation in all plant parts. Gene *F* controls the distribution of anthocyanins to the flower, gene *E* distributes the pigment to the periderm of the tuber with deep colour around the eyes and gene *M* restricts pigmentation of tuber periderm to areas around the eyes (Howard, 1962; 1964; 1969; de Jong, 1991). Complementary genes *C* and *I*, with a dominant inhibitor *Z*, have been suggested for distribution of pigment to tuber flesh (Howard, 1969).

Work has also been carried out with diploid and tetraploid potatoes on the association of genes with a specific linkage group and to assign the linkage groups to specific chromosomes (Kessel and Rowe, 1974), and more recently to map the loci included in anthocyanin inheritance by RFLP (van Eck *et al.*, 1993; 1994).

1.8 Reasons for study and outline of thesis

Potatoes are an important food source worldwide, and consumers are becoming increasingly concerned with the appearance of food and the use of natural food colourants. For many centuries white coloured potatoes have been preferred, but recently an interest towards the coloured varieties has occurred. Novelty chips made from purple fleshed varieties are being produced commercially in the USA, and potato salads using a mixture of white and purple fleshed tubers are proving popular with restaurants and airlines. The major selling point for these products is that the colour is natural and is not an additive.

Coloured potatoes may also prove to be an important source of anthocyanins for natural food colourants. They have a high production of anthocyanin per hectare and may become an important source of the red coloured anthocyanins, such as Pg and Pn, which are not produced by grapes (which are currently a major source of anthocyanin food colourants).

Potato tubers are also of interest to plant physiologists because they produce their anthocyanin underground in the dark, whereas many flowers and fruits will not produce anthocyanins unless exposed to light. It is also interesting that many "white" tubers will produce anthocyanins only after harvest and exposure to light (Black, 1933).

On the basis of the above, it was of interest to investigate the biochemistry of anthocyanins in potatoes, including the biosynthetically closely related classes of flavonoids and phenolic acids. Any investigation would not be complete without a full study of the precursors and related compounds to provide a complete picture of the biochemical pathways. This thesis is divided into a number of chapters. Chapter 2 is involved with food colour and the effect of carbohydrates on the colour of anthocyanins. Chapter 3 is concerned with the identification of anthocyanins, flavonoids and phenolic acids present in potatoes, and a survey of these compounds from a number of *S. tuberosum* cultivars and *Solanum* species was carried out. Composition and concentration of phenolics from the different *S. tuberosum* cultivars and *Solanum* species, and different plant organs were compared, and related to the anthocyanin

concentration, to estimate the flow of metabolites through the various pathways (e.g. anthocyanin, flavonol, etc). Studies of the enzymes involved in flavonoid biosynthesis in tubers were carried out, and the differences between white, red and purple coloured tubers examined. Changes in anthocyanin, flavonoid and phenolic acids concentrations during the development and storage of the tuber were studied in Chapter 4, together with the distribution of anthocyanin pigments within the tuber. The biosynthesis of the flavonoids was investigated further in the fifth chapter. Tissue-cultured minitubers were used to study the effect of light on flavonoid biosynthesis and enzyme activities, and attempts were made to answer the question of "Why do tubers produce anthocyanins in the dark?". In the sixth chapter, studies on the location of anthocyanin pigmented cells in various coloured cultivars were carried out. The seventh chapter details a number of experiments designed to investigate the effect of various cooking procedures on the final colour of purple fleshed potatoes, with a final, overall concluding discussion in Chapter 8.

CHAPTER 2

The role of anthocyanins in food colour and the effect of carbohydrates on anthocyanin colour

2.1 Introduction

Three major factors which influence consumer preference in food and food products are colour, flavour and texture. Others factors include price, freshness, packaging, impulse buying, etc. Colour is one of the most important attributes, both for its intrinsic, aesthetic value and its value as an indicator of food quality. Food selection or judgement of quality would be extremely difficult if colour discrimination was removed. By the process of experience, people learn what colours to expect and consider natural and desirable, and may be able to predict what properties a food or beverage will have from their knowledge of similar materials.

2.1.1 Factors affecting food colour

Anthocyanins are important in the appearance of many fruits, red wines, fruit juices, jams, some vegetables, and many other food products. Many factors affect the colour of anthocyanins and therefore food colour. Some of the most important factors are discussed below.

2.1.1.1 Nature of the anthocyanin

The degree of hydroxylation and methoxylation of the B-ring affects the colour of anthocyanins. As the number of hydroxyls increases, the colour becomes bluer, and the substitution of these hydroxyl groups with methyl groups reverses the trend to a darker red colour (Figure 2.1). Hydroxyls attached to the A- and C-rings of the anthocyanin molecule are also important in determining colour (Stafford, 1990). Glycosylation and acylation may also affect the colour of anthocyanin pigments to some degree, but are usually more important in providing stability to the molecule. Anthocyanin molecules tend to be unstable, but glycosylation of the hydroxyl at position 3 on the C-ring increases stability, which is possibly why all anthocyanins, *in vivo*, are glycosylated at this position. Anthocyanins containing two or more aromatic acyl groups are extremely stable, whereas monoacylated anthocyanins do not display such a degree of colour

stability. Brouillard (1982) has suggested that the presence of hydrophobic interactions between the flavylium C-ring and the aromatic portions of the acyl groups protects the flavylium ring from nucleophilic attack by water, and proposed that in anthocyanins containing two acyl groups, one acyl group is situated above the flavylium ring and the other beneath it.

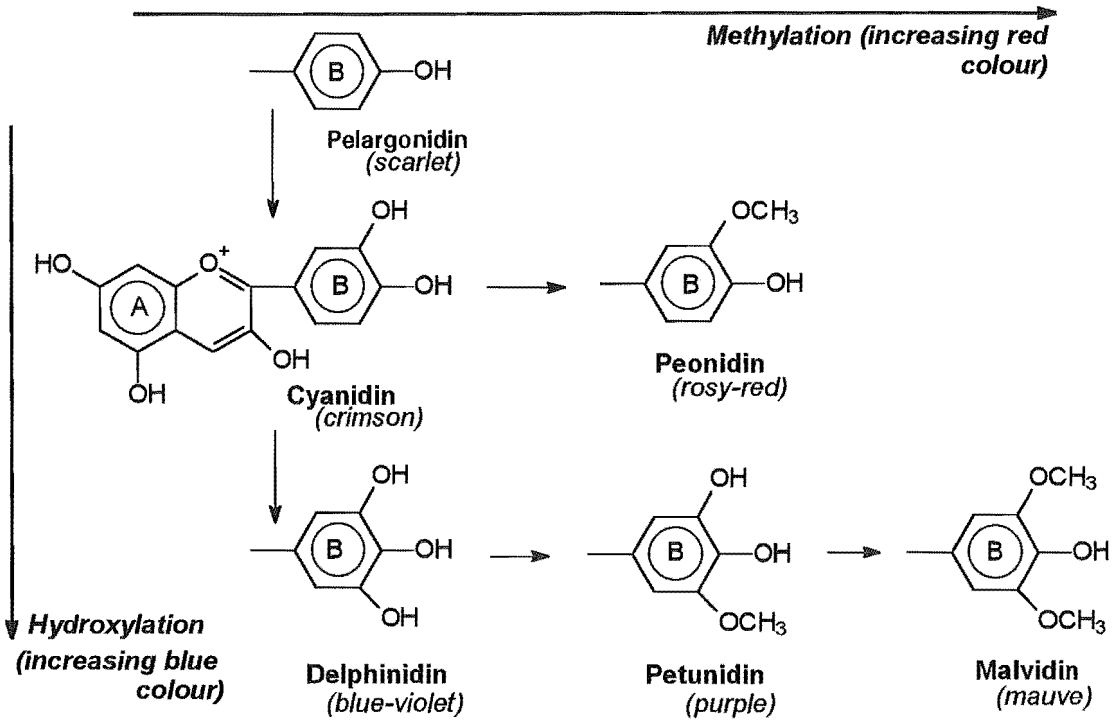


Figure 2.1 Effect of hydroxylation and methylation of the B-ring on the colour of anthocyanins.

2.1.1.2 pH

pH has a large effect on the colour of anthocyanins (Figure 2.2) because the three species of water (*i.e.* H^+, OH^- and H_2O) are highly reactive towards anthocyanins. Thus, the solvent (water) plays an important role in influencing both the stability and reactivity, as well as the spectral properties of the various structures adopted by anthocyanins in aqueous solutions. In acidic aqueous solutions, four species of anthocyanin molecule may exist in equilibrium:- the quinonoidal base A, the flavylium cation AH^+, the pseudobase (or carbinol) PB, and the chalcone C (equations (i)-(iii)) (Brouillard, 1982; Chen and Hrazdina, 1982).

				equation
AH^+	$\Leftrightarrow$	$A + H^+$	acid/base equilibrium	(i)
$AH^+ + H_2O$	$\Leftrightarrow$	$PB + H^+$	hydration equilibrium	(ii)
PB	$\Leftrightarrow$	C	ring/chain tautomeric equilibrium	(iii)

Figure 2.2 Effect of changing pH from pH 1.5 to pH 5.0 on the colour of delphinidin-3,5-diglucoside.

Inter-conversion between these structures (A, AH^+ and PB) may take place as shown in Figure 2.3 below.

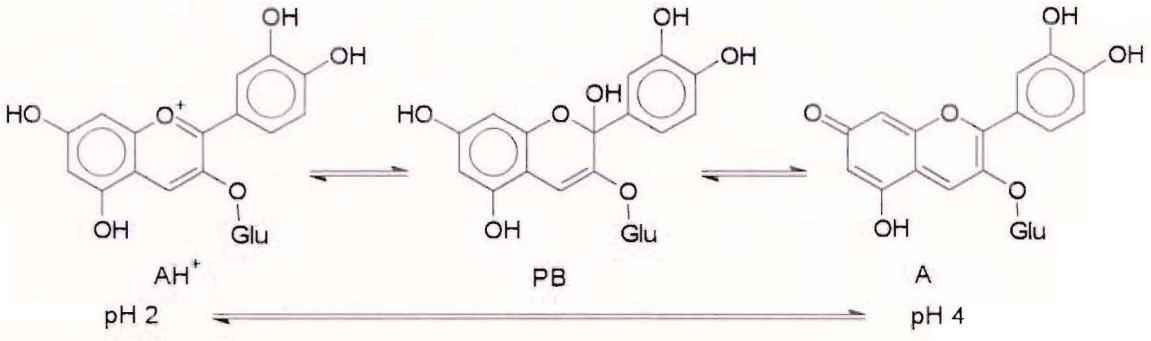


Figure 2.3 Effect of pH upon the structure of cyanidin (after Brouillard, 1982). Only reactions occurring at acidic pH (equations i and ii) are considered here. A = quinonoidal base, AH^+ = flavylium cation and PB = pseudobase.

In most higher plants the pH of a mature vacuole is acidic, and a survey of many flowers has shown that the pH of the vacuole ranges from 2.5-7.5 (Stewart *et al.*, 1975). For the most acidic cells, pigmentation is probably due to the highly coloured flavylium form (AH^+) alone, whereas in the range pH 3-4, both the flavylium cation and the neutral tautomer (A) may contribute to colour, whilst at pH 4-6 the neutral tautomers dominate because of deprotonation of the flavylium cation (equation i) (Brouillard, 1982). Thus, pH is a major factor in the production of anthocyanin colour (Brouillard, 1982; Chen and Hrazdina, 1982).

2.1.1.3 Copigmentation

At the pH of cell vacuoles, anthocyanins may form hydrogen bonds with other molecules which cause an increase in the colour of the anthocyanin, and a bathochromic shift of the visible λ_{max}, thus resulting in the "blueing" of red shades. These molecules are called copigments and often have little or no colour by themselves. Copigments are often phenolic in nature and include flavonoids, polyphenols, alkaloids, amino acids, organic acids and anthocyanins themselves. The mechanism of copigmentation has been reviewed in detail by Brouillard (1983) and Brouillard *et al.* (1989). Copigments are thought to act by protecting the flavylium ring against attack by water, and the removal of water displaces the hydration/dehydration equilibrium towards the coloured species (AH^+, equation ii). The intensity of the copigment effect has been shown to be dependent upon both the type and concentration of the anthocyanin and copigment, the pH of the medium, temperature, and solvent (copigmentation only occurs in aqueous solutions) (Mazza and Brouillard, 1990).

2.1.1.4 Self-association

Self-association of anthocyanins is a form of copigmentation occurring between anthocyanin molecules in solution. It was first postulated by Asen *et al.* (1972) to explain why the absorbance of a cyanidin solution deviated from the Beer-Lambert Law with increasing concentration. Vertical stacking occurs in anthocyanins, especially those with sugars at both the 3 and 5-positions, and this protects the flavylium ion from hydrolytic attack (Harborne, 1988). Therefore, as a result of this self-association, the colour may increase more than proportionally to pigment concentration and the typical bathochromic shift of copigmentation is also observed (Timberlake, 1980).

2.1.1.5 Metal complexing

Anthocyanins may also form complexes with metals, which causes similar bathochromic shifts as copigmentation. However, metal complexes can only occur in those anthocyanins containing *o*-dihydroxyl groups (cyanidin (Cy), delphinidin (Dp), and petunidin (Pt)) (Jackman *et al.*, 1987). Magnesium (Mg^{2+}) has been found to be involved in the pigment complex, commelinin, from blue flowers of *Commelina communis* (Osawa, 1982), and the blueing of the red sepals of *Hydrangea macrophylla* has been shown to be the result of the complexing of the anthocyanin with aluminium (Harborne, 1988). Jurd and Asen (1966) found that in the presence of metal ions (e.g. Al^{3+}), chlorogenic acid and quercetrin acted as copigments in anthocyanin solutions whereas, in the absence of metals, chlorogenic acid and quercetrin had no measurable effect on the colour or stability of the anthocyanin. The addition of copper, iron, tin and aluminium ions to cranberry juice provided greater stability for anthocyanins (Starr and Francis, 1973). The relatively good stability of metal-anthocyanin complexes has led to the suggestion that they be used as food colourants (Francis, 1989), however the overall colour changes produced with metal ions are often undesirable, with green or brown colours produced in the reaction of the metal ions with other food substituents (Starr and Francis, 1973).

2.1.2 Factors affecting the degradation of anthocyanins in foods

There are a large number of factors which reduce the colour of anthocyanins and therefore affect food colour. A brief review of some of these factors is given below.

2.1.2.1 Temperature

The effect of temperature on anthocyanins has been well studied because of its obvious importance to the quality of food. All studies have found that anthocyanins show a rapid decrease at high temperatures (reviewed in Francis, 1989). Markakis *et al.* (1957) reported that pigments in strawberry preserves had a half-life of only one hour at 100°C, and recommended a short time/high temperature process as best for pigment retention when processing foods. The first step in the thermal degradation of anthocyanins is the removal of the glycosidic sugars by hydrolysis, followed by the degradation of the anthocyanidin (Timberlake and Bridle, 1966). It has been suggested that the path of thermal degradation of anthocyanins is via the chalcone, and Brouillard (1982) found that higher temperatures favoured the conversion of the anthocyanin to the open ringed chalcone and, after ring opening, subsequent degradation of the chalcone lead to the formation of brown products.

2.1.2.2 Sugars and their degradation products

The role of free sugars in the medium on anthocyanin degradation is very complex, but generally the presence of sugars increases the degradation of anthocyanins over time. Meschter (1953) was the first to show that, in the presence of sugars, the rate of pigment breakdown was associated with the rate at which the sugar was degraded to furfural-type compounds. These compounds, furfural (from aldo-pentoses) and 5-hydroxy-methylfurfural (HMF, from keto-hexoses), may be formed via the Maillard reaction, or by the oxidation of ascorbic acid, polyuronic acids, or even anthocyanins themselves (Jackman *et al.*, 1987). Other workers (Daravingas and Cain, 1968; Debicki-Pospisil *et al.*, 1983) also found that furfural and HMF accelerated the rate of anthocyanin degradation and may condense with anthocyanins to form complex brown coloured compounds.

2.1.2.3 Ascorbic acid and oxygen

Ascorbic acid and anthocyanins interact causing the concurrent disappearance of both compounds which reduces both the appearance and nutritive value of foods. It is thought that the oxidative products of ascorbic acid, rather than ascorbic acid itself, are important in anthocyanin destruction (Jackman *et al.*, 1987). The presence of O_2 also accelerates the degradation of anthocyanins and it has been shown that the preparation of food products under N_2 or vacuum reduced the anthocyanin decomposition (Francis, 1989). O_2 may cause deleterious effects on anthocyanins through a direct oxidative mechanism and/or through indirect oxidation of other constituents in the medium (e.g. ascorbic acid) which are then capable of reacting with the anthocyanins to yield colourless or brown products (Jackman *et al.*, 1987).

2.1.2.4 Enzymatic degradation

Anthocyanins can be degraded by an number of enzymes found in plant tissue including glycosidases and diphenol oxidases (DPO's; phenolases) (Francis, 1989). Glycosidases, sometimes called anthocyanases, hydrolyse the anthocyanins to sugars and anthocyanidins, which are unstable and degrade to colourless derivatives. DPO enzymes are found throughout the plant kingdom and, in the simplest example, oxidise *o*-dihydroxyphenols to *o*-quinones. The quinones may react with each other or a number of other compounds, such as proteins or amino acids, to form brown polymers (Francis, 1989). DPO's can react directly with anthocyanins, but the reaction is usually more pronounced when other phenolics, which are better substrates for this enzyme, are also present, and a system of sequential co-oxidation reactions, resulting in anthocyanin

degradation, is thought to occur (Peng and Markakis, 1963). Glycosidases and DPO's can be inactivated in a number of ways, including blanching (Siegal *et al.*, 1971; Wrolstad *et al.*, 1980), inhibition by a range of inhibitors, for example, sulphur dioxide, sodium metabisulphite, dithiothreitol, phenylhydrazine, and cysteine (Cash *et al.*, 1976; Siddiq *et al.*, 1994; Walker, 1995).

2.2 Effect of carbohydrates on anthocyanin colour

During the present work with coloured potato tubers it was noticed that extracts of anthocyanins often decreased in colour after extraction. Figure 2.4 shows the decrease in colour occurring over a 2min period after the extraction solvent (15% acetic acid in methanol) was added to ground Urenika tuber tissue. This decrease was in marked contrast to flower (*Camellia*, *Delphinium*, mallow) extracts, and it was thought that the high levels of starch present in tubers might be responsible for this effect. Because many natural pigments in plant foods co-exist with a variety of saccharides, which may affect the structure and stability of the pigments, these saccharides could play a role in the final colour of food products. Therefore, it was considered of interest to investigate more fully the effect of various carbohydrates, including monomers and polymers, on the spectral properties of anthocyanins.

2.2.1 Review of carbohydrates used in study

Some relevant properties of the selected plant and food carbohydrates used in this study are summarised below.

In most plants starch contains about 20-25% amylose (AM) and 75-80% amylopectin (AP). AM consists of essentially linear chains of α-(1,4) linked glucose with occasional α-(1,6) branch points (Morrison and Karkalas, 1990). AM exists as a helical structure (Figure 2.5a), with six successive glucose units per revolution and a 5Å wide cavity in the centre of the helix, and therefore it can form inclusion complexes with a number of compounds such as, alcohols, fatty acids and iodine (Teitelbaum *et al.*, 1978; 1980). AP (Figure 2.5b) consists of α-(1,4)-glucan chains joined by numerous α-(1,6)-branch points which disrupt helix formation, and only the short linear chains are capable of forming helices (Kennedy and White, 1979).

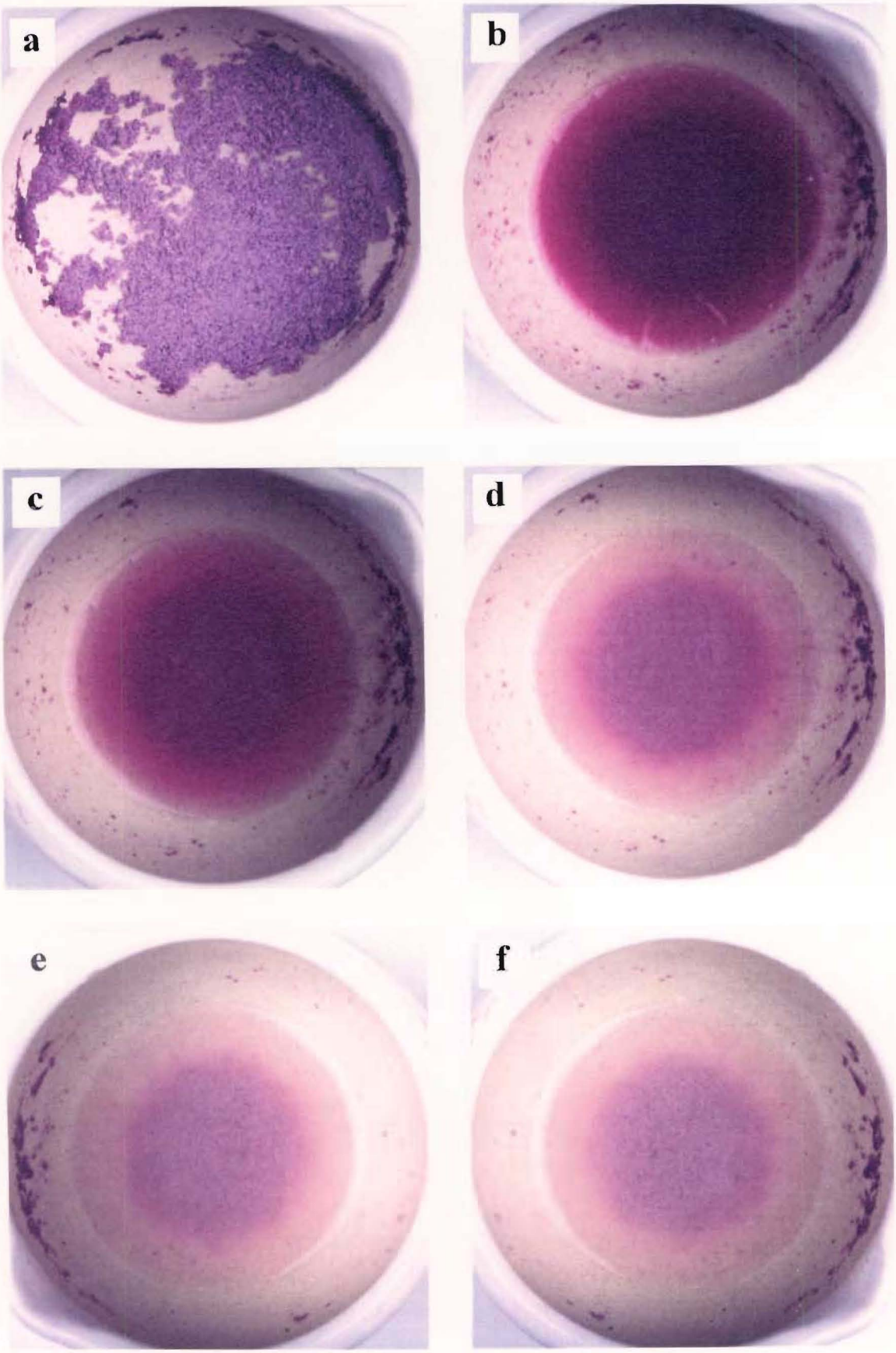


Figure 2.4 Decrease in colour of ground Urenika tuber tissue at a) powder ground in liquid N_2, before extraction solvent was added, b) 5-10s, c) 20s, d) 40s, e) 60s and f) 120s, after the acidic methanol extraction solvent was added.

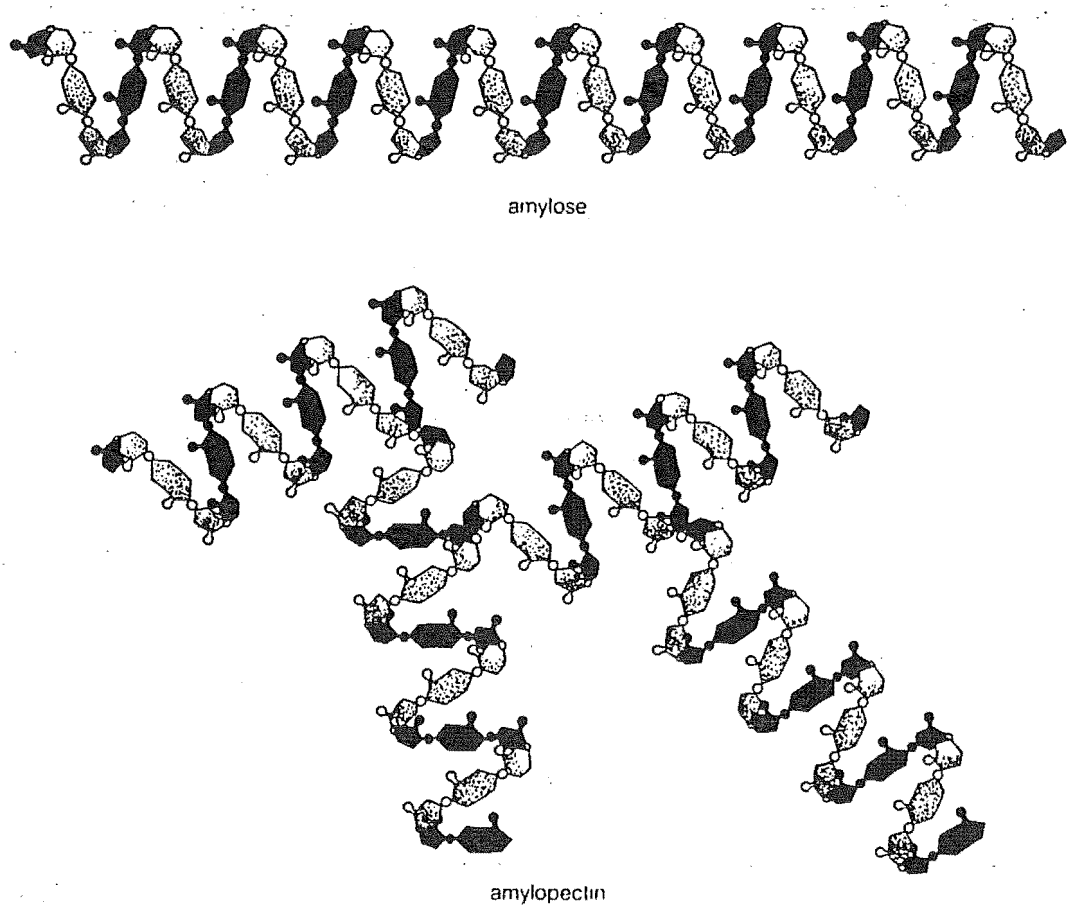
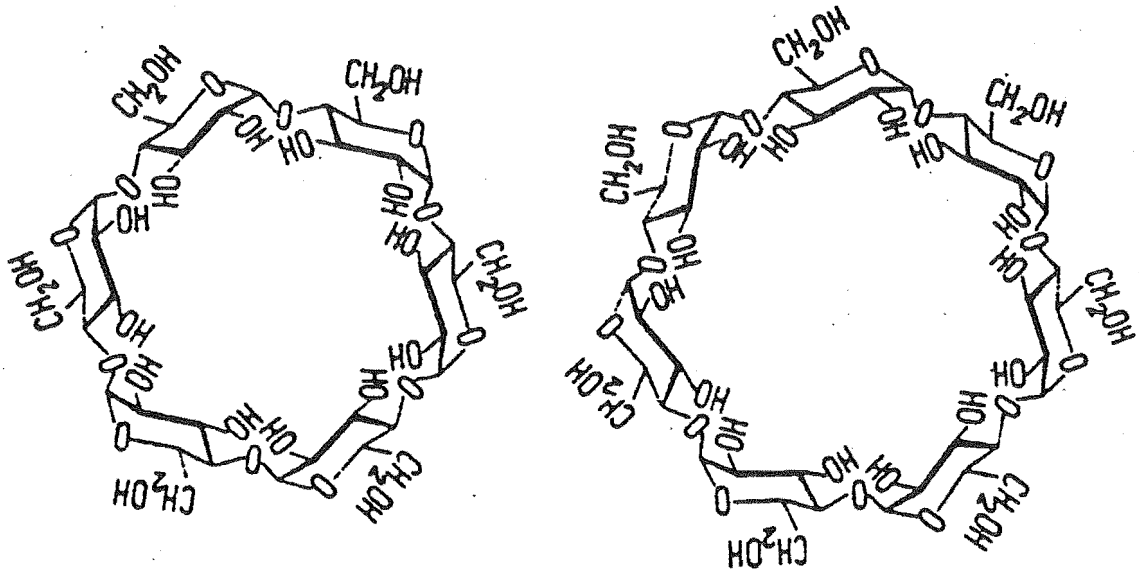


Figure 2.5 A schematic representation of a small part of amylose and amylopectin (from Salisbury and Ross, 1992).

Cyclodextrins (CDs) are cyclic oligosaccharides containing α-(1,4)-linked glucose units; the most common are α-cyclodextrin (α-CD) (cyclohexaamylose) and β-cyclodextrin (β-CD) (cycloheptaamylose) which have 6 and 7 glucose units respectively (Figure 2.6) (Saenger, 1980; Brouillard *et al.*, 1989; Chandra *et al.*, 1993). The less common γ-cyclodextrin, containing eight glucose units, was not used in this study. In solution, a cyclodextrin (CD) molecule has a tubular form with an inner diameter of 6Å for α-CD and 7.5Å for β-CD. They can form reversible inclusion complexes with smaller molecules (often phenolic substances) which fit into the cavity (in both the solid and liquid state). Irwin *et al.* (1994) found that both α- and β-CD formed inclusion complexes with chlorogenic acid, a major phenolic acid found in potato tubers. CD's are known to bind readily to molecules regardless of solvent environment, which is in marked contrast to copigmentation effects which occur only in aqueous systems (Brouillard *et al.*, 1989; Chandra *et al.*, 1993).



α - cyclodextrin β - cyclodextrin

Figure 2.6 Structures of α- and β-cyclodextrin (from Szejtli, 1978).

Pectins are major components of plant tissues, comprising a substantial proportion of the parenchyma of fleshy roots (Coultate, 1989) and represent 0.36% of the fresh weight of potato tubers (Robinson, 1987). Pectin is a mainly linear polymer composed of esterified galacturonic acid residues, linked by α-(1,4) glycosidic bonds (Belitz and Grosch, 1987; Robinson, 1987).

Polydextrose (PolyD) is a synthetic molecule composed almost entirely of randomly cross-linked glucose polymers with α-(1,6) bonds predominating. It is used in foods as a reduced calorie, bulking and texturising agent, and as a substitute for sugar and fat (Leibrand *et al.*, 1985).

2.3 Materials and methods

2.3.1 Preparation of anthocyanins

A range of anthocyanins were prepared from freeze-dried strawberries (*Fragaria ananassa*) (pelargonidin-3-glucoside (Pg-3-glu)), *Delphinium* spp petals (delphinidin-3-

glucoside (Dp-3-glu) and delphinidin-3,5-diglucoside (Dp-3,5-glu)), fresh mallow (*Malva sylvestris*) petals (mainly malvidin-3,5-diglucoside (Mv-3,5-glu)) and potato (*S. tuberosum*) tubers, cultivars Red Flesh (mainly pelargonidin-3-*p*-coumaroyl-rutinoside-5-glucoside (Pg-RF) and Urenika (mainly malvidin-3-*p*-coumaroyl-rutinoside-5-glucoside (Mv-U)). Plants tissues were extracted with 15% acetic acid in methanol and the anthocyanins chromatographed on a column of C_{18}, eluted with 60% (v/v) methanol, evaporated to dryness *in vacuo*, and re-dissolved in H_2O. The pH of the anthocyanin solutions was adjusted (with HCl or NaOH) to pH 2 and pH 4, giving a final absorbance (at λ_{max}) of 0.6 to 1.0 at pH 2, or 0.4 to 0.8 at pH 4.

Two anthocyanin standards, cyanidin-3-rutinoside (Cy-3-rut) and malvidin-3-glucoside (Mv-3-glu) were obtained from Plantech (UK). The anthocyanins used in this study are listed in Table 2.1.

Table 2.1 Source of anthocyanins.

Anthocyanin	*Source*	λ_{max} *(nm)*	
		pH=2	*pH=4*
Cy-3-rut	standard (Plantech, UK)	512	518
Dp-3,5-glu	delphinium flowers	534	568
Dp-3-glu	delphinium flowers	536	570
Mv-3-glu	standard (Plantech, UK)	518	526
Mv-3,5-glu	mallow flowers	540	546
Pg-3-glu	strawberry fruit	498	498
Pg-RF	potato tubers, cv. Red Flesh	504	518
Mv-U	potato tubers, cv. Urenika	524	538

2.3.2 Preparation of carbohydrates

Solutions of the following carbohydrates were prepared by dissolving in H_2O followed by adjustment to pH 2 or 4: 1.5% AP, 3% α-CD, 3% β-CD, 1.5% pectin, 3% PolyD, 50% sucrose, 50% glucose, 50% fructose, and 50% maltose (all as w/v). Solutions of 1.5% AM were prepared by dissolving initially in 50% (w/v) KOH and then the pH was adjusted with HCl (with the final concentration of KOH being 12.5%). All reagents, except PolyD (Pfizer, USA), were supplied by Sigma (USA).

2.3.3 Spectrophotometric assay

For the spectrophotometric assays, equal amounts (250μl) of anthocyanin and carbohydrate solutions were mixed and the spectra (blanked against carbohydrate

solution) recorded after 60min. Controls (without added carbohydrate) contained 250µl anthocyanin plus 250µl distilled H_2O (or KOH/HCl blank for AM) (at pH 2 or 4). Results are presented as a percentage of the control absorption at its visible λ_{max}. Spectra were recorded on a Hewlett Packard 8452A diode-array spectrophotometer.

2.4 Results

The purity of the two anthocyanin standards, cyanidin-3-rutinoside (Cy-3-rut) and malvidin-3-glucoside (Mv-3-glu), was confirmed by HPLC (refer to Section 3.3.3.2 for method). Each plant extract was also analysed by HPLC and found to contain one or two major anthocyanins, and also phenolic acids and other flavonoids. The phenolic acids and other flavonoids (besides anthocyanins) did not appear to have any obvious effect on the results, because similar reactions with the carbohydrates were found whether using anthocyanin extracts, containing flavonoids and phenolic acids, or the pure anthocyanins.

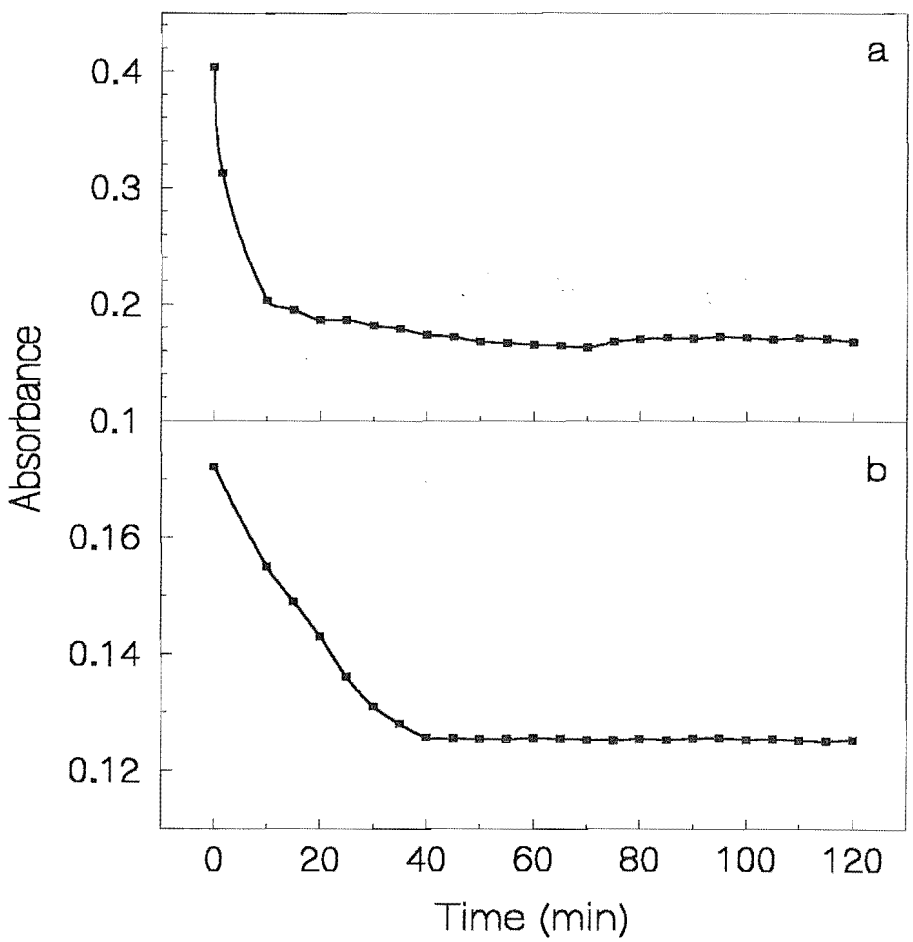


Figure 2.7 Time course of colour disappearance after addition of β-cyclodextrin (β-CD) to a) malvidin-3,5-diglucoside and b) cyanidin-3-rutinoside.

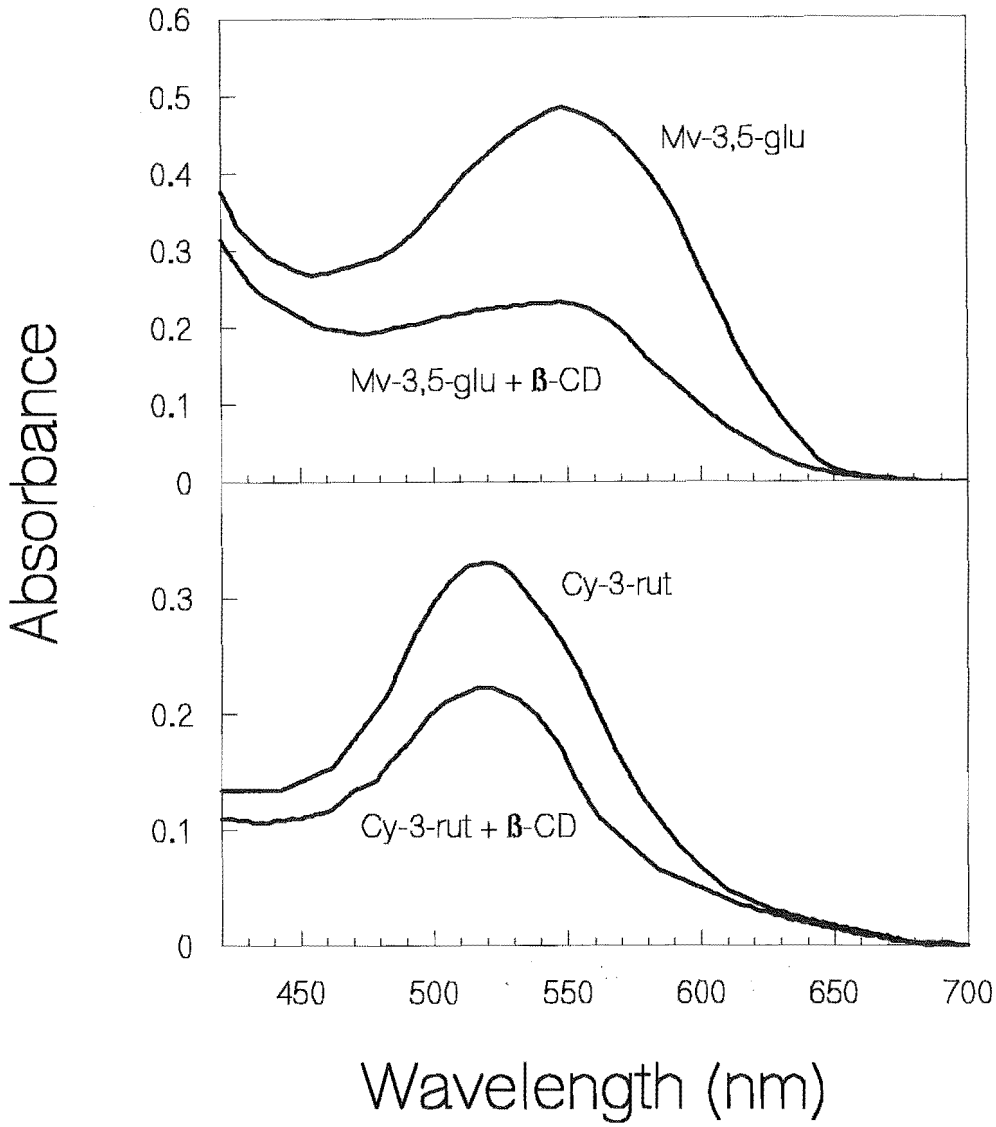


Figure 2.8 Spectra of malvidin-3,5-diglucoside (Mv-3,5-glu) and cyanidin-3-rutinoside (Cy-3-rut) at pH 4.0 in presence or absence of β-cyclodextrin (β-CD).

The loss of colour was time-dependent, and the rate of colour loss was dependent on the identity of the anthocyanin. This was shown, for example, by the difference in the rate of colour loss after the addition of β-CD to Mv-3,5-glu and Cy-3-rut, where the addition of β-CD to Mv-3,5-glu initially showed a rapid decrease in colour followed by a much slower decrease (Figure 2.7a) whereas, when β-CD was added to Cy-3-rut, the absorbance decreased almost linearly to 30-40min before levelling out and becoming stable (Figure 2.7b). For all anthocyanins the resultant colour was stable after 60min. As examples, representative spectra showing Cy-3-rut (standard) and mallow extract (mainly Mv-3,5-glu), both without (control) and with β-CD are given in Figure 2.8

which shows the decrease in absorbance at 60min after the addition of β-CD. There was a significant decrease in absorbance in the visible region, but there was no change in the λ_{max} value of either pigment. Figs 2.9a-e show the effect of added AM, AP, α-CD, β-CD and glucose on a range of anthocyanins at pH 2 and 4. The other anthocyanins tested (summarised in Table 2.2) displayed similar reactions to these, with the same aglycone as those tested, after addition of the carbohydrate.

In the presence of AM, α-CD and β-CD there were significant decreases in absorbance at the visible λ_{max} at pH 4, compared with that in the absence of these carbohydrates (control), although the magnitude of the decrease varied, depending on the identity of the carbohydrate and also the anthocyanin (Figure 2.9a, c and d). The effect of added AP at pH 4 depended on the identity of the anthocyanin (Figure 2.9b). The addition of AM, AP, α-CD and β-CD to the anthocyanins at pH 2 all caused a decrease in colour, compared with that in the absence of added carbohydrate (control), but this decrease in colour (as a percentage of the control) was typically smaller at pH 2 than that which occurred at pH 4 (Figure 2.9a-d). By contrast to these polysaccharides, the addition of glucose resulted in an increase in absorbance at pH 4, and only a small increase or no significant change at pH 2 (Figure 2.9e). The results for sucrose (not shown) were similar to the results presented in Figure 2.9e for glucose, whilst maltose (not shown) also showed only a small increase or no significant change at pH 2, but showed twice the increase compared with that of glucose at pH 4. Results with all carbohydrates are summarised in Table 2.2. Polydextrose and fructose showed no significant effect at either pH, whilst pectin showed a change only at pH 4. Essentially similar results were obtained with anthocyanins from different sources. The degree of colour change appeared to be related primarily to the identity of the anthocyanin aglycone and only secondarily to its sugar substitution and/or acylation pattern, since similar changes in colour were found for anthocyanins with the same aglycone, but not those with similar sugar and acylation substitution patterns (Figure 2.9 and Table 2.2). However, further work needs to be carried out to confirm this because only a limited number of anthocyanins were used in this study.

In all cases there was no change in λ_{max}, only a change in the intensity of absorbance. The fading effect was dependent on both the anthocyanin and carbohydrate concentration. For example, increasing anthocyanin concentration (Mv-3,5-glu) caused a smaller proportional decrease in absorbance compared with the control (Figure 2.10), and increasing β-CD concentrations caused a larger decrease in absorbance of Mv-3,5-glu (Figure 2.11a) and Cy-3-rut (Figure 2.11b).

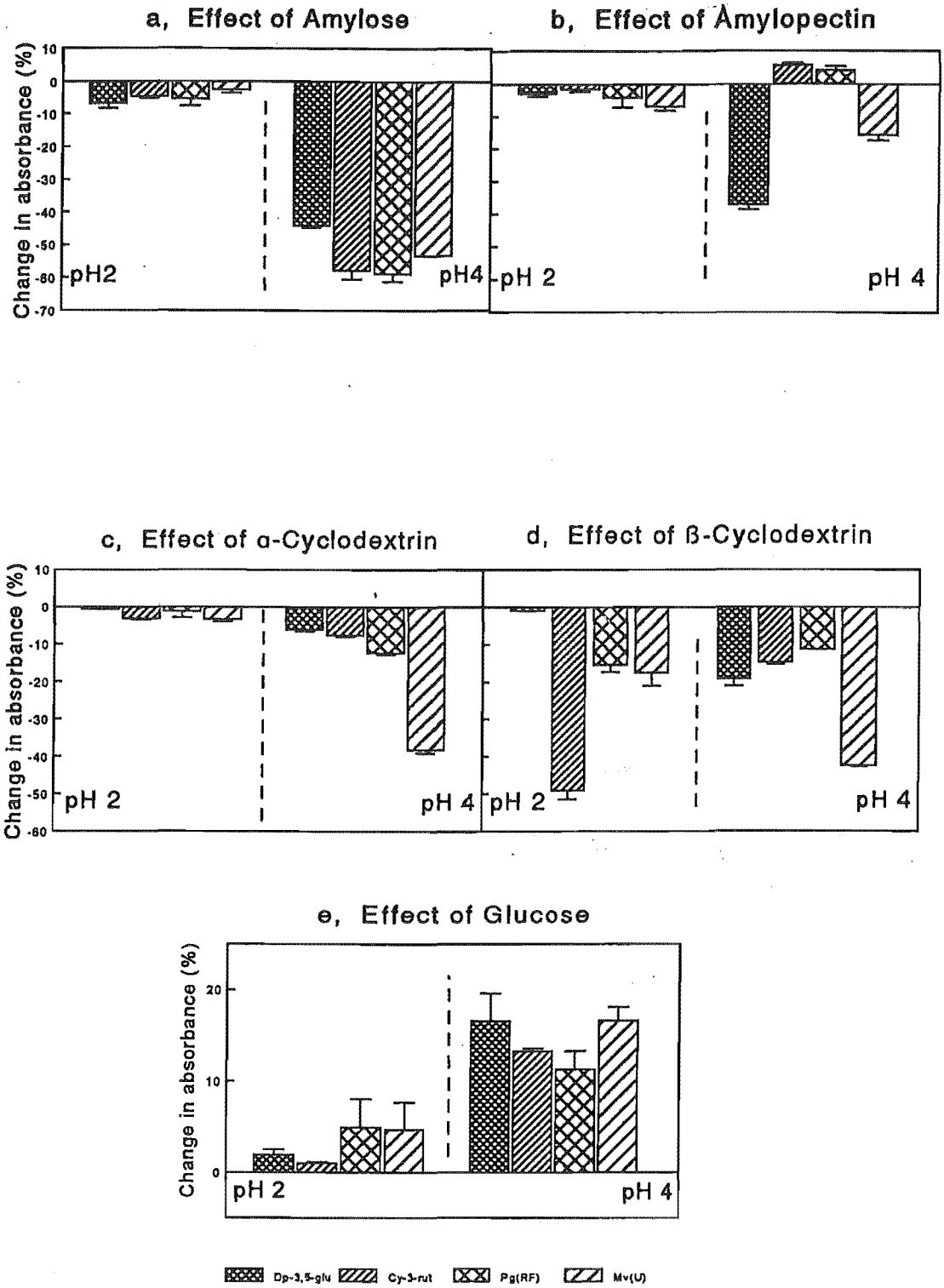


Figure 2.9 Effect of added carbohydrate on colour of anthocyanins at pH 2 or 4. a) amylose (AM), b) amylopectin (AP), c) α-cyclodextrin (α-CD), d) β-cyclodextrin (β-CD) and e) glucose. Results are presented as % change in absorbance at λ_{max}.
Error bars represent ± 1 standard error.

Table 2.2 Change in anthocyanin colour (as ΔA% compared with control) in the presence of carbohydrate.

Carbohydrate	ΔA at pH 2	ΔA at pH 4
Amylose*	3-7 % decrease	45-58% decrease
Amylopectin*	2-7% decrease	Dp 37% decrease Cy and Pg 0-5% increase Mv 15% decrease
α-cyclodextrin*	no change	5-35% decrease
β-cyclodextrin*	0-50% decrease	10-45% decrease
Polydextrose	no change	no change
Pectin	no change	Dp 20% decrease Cy, Pg and Mv 10-20% increase
Sucrose	0-4% increase	10-18% increase
Glucose*	0-5% increase	10-18% increase
Fructose	no change	no change
Maltose	0-5% increase	20-45% increase
KOH/HCl	no change	0-50% increase

* see Figs 2.9a-e for more detail
Cy = cyanidin
Dp = delphinidin
Mv = malvidin
Pg = pelargonidin

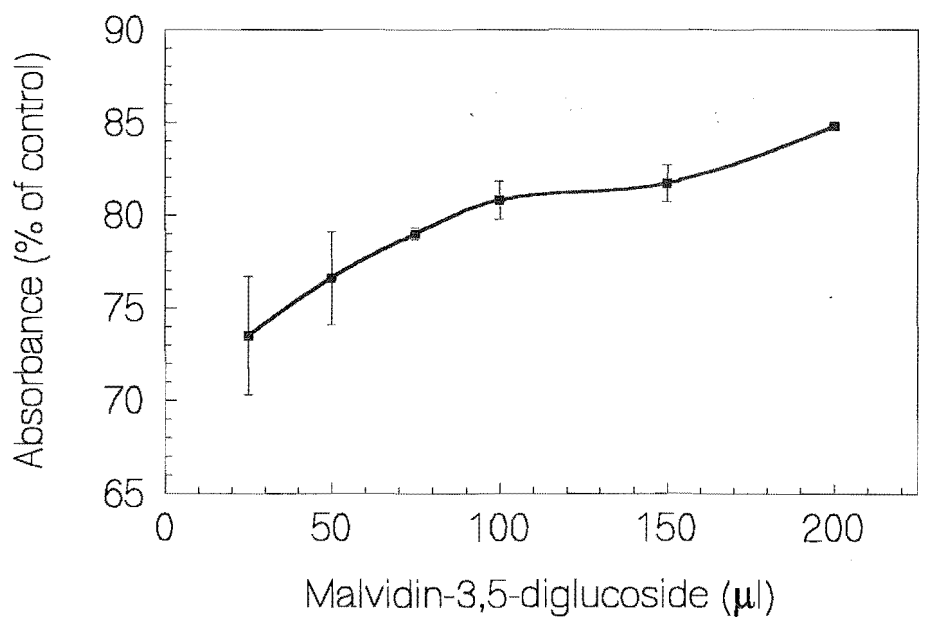


Figure 2.10 Effect of increasing concentrations of malvidin-3,5-diglucoside extract on the colour produced in a reaction with β-cyclodextrin (β-CD) at pH 4. Error bars represent ± 1 standard error. Each assay solution contained 250μl β-CD, plus the stated amount of malvidin-3,5-diglucoside extract, with each assay solution made up to 500μl with water at pH 4. Controls contained water at pH 4 instead of β-CD (at each anthocyanin concentration).

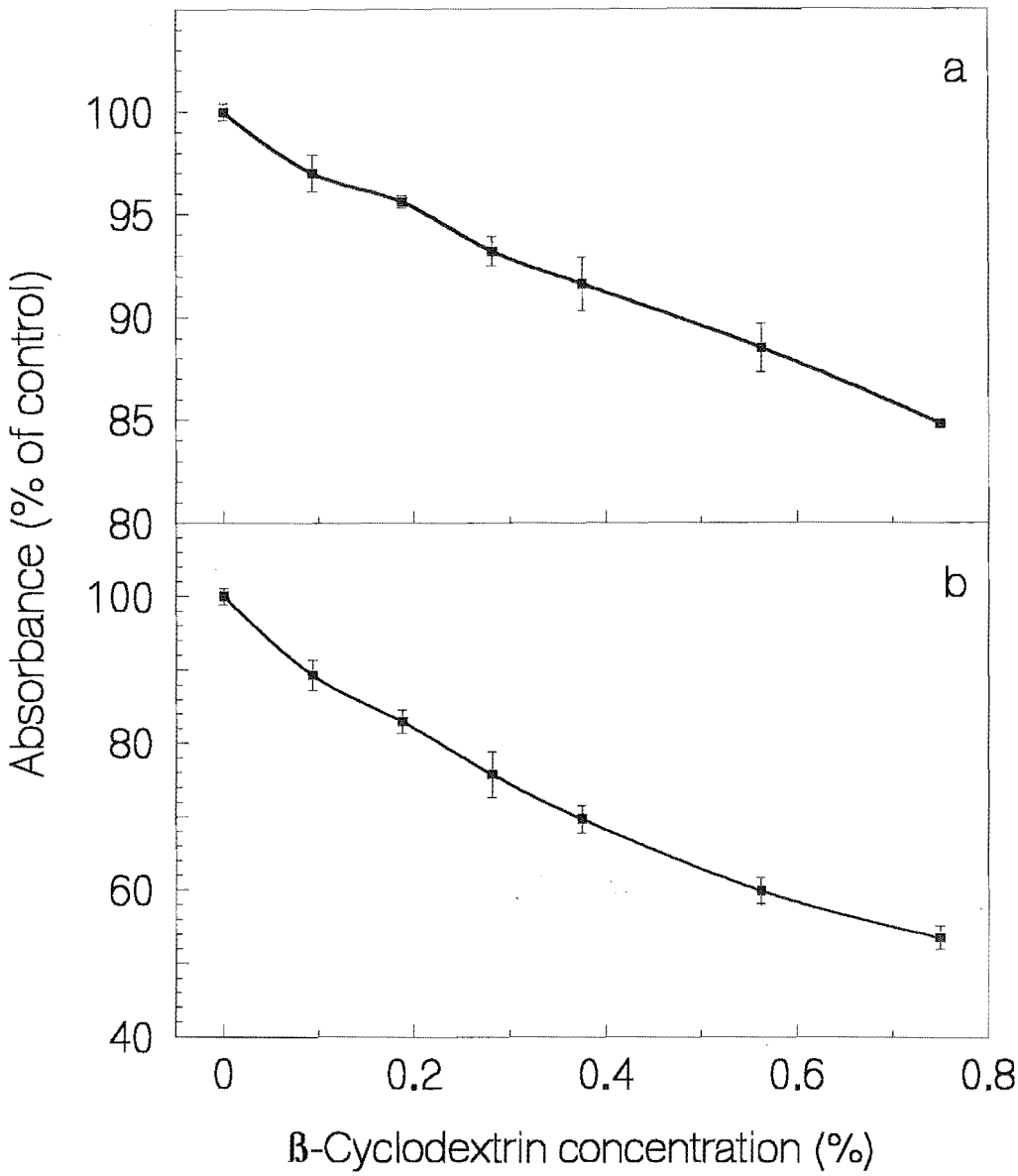


Figure 2.11 Effect of increasing β-cyclodextrin (β-CD) concentrations at pH 4 on a) malvidin-3,5-diglucoside extract and b) cyanidin-3-rutinoside standard. Error bars represent ± 1 standard error. Each assay solution contained the anthocyanin plus the stated concentration of β-CD. Controls contained water at pH 4 instead of β-CD.

2.5 Discussion

From Table 2.2 and Figure 2.9 a-e it may be seen that the addition of AM, AP, α-CD and β-CD resulted in significant decreases in anthocyanin colour, with AM and β-CD showing the largest effects. The α-CD molecule has a smaller inner diameter than β-CD and appeared to be less efficient at forming inclusion complexes with anthocyanins. AP is a much more highly branched molecule than AM and can only form helical structures within the unbranched straight chains of the molecule. Therefore, if the colour decrease was caused by the inclusion of the anthocyanin within the helix, the lower number of helices formed by AP may account for the smaller decrease in anthocyanin colour shown when AP was added compared with when AM was added.

The "fading" effect of anthocyanins with added α-CD and β-CD has also been reported by Yamada *et al.* (1980) who examined the interaction of these CD's with three anthocyanins [Pg-3-glu, Cy-3-glu and Dp-3-(4-(*p*-coumaroyl)-L-rha-(1,6)-glu)-5-glu]. In their experiments, each anthocyanin was adjusted to pH 2 and added to varying concentrations of α- or β-CD. In summary, their addition of β-CD resulted in the fading of two anthocyanin solutions (Pg-3-glu and Cy-3-glu), and this effect was increased with higher concentrations of β-CD. Addition of α-CD resulted in a fading effect only for Pg-3-glu and this was less than that measured for β-CD. No change in λ_{max} was observed. Yamada *et al.* (1980) also investigated the effect of amylose (dissolved in 33% DMSO) on these anthocyanins, and found that the addition of AM only affected the fading of Pg-3-glu, but the extent of this effect could not be determined due to immediate AM precipitation. The precipitation of AM from DMSO solutions was also observed in our experiments, but was overcome by initially dissolving AM in 50% KOH and then re-adjusting the pH to 2 or 4. This procedure resulted in the AM remaining in solution and retaining its helical structure, as shown by its characteristic reaction with KI/I_2 solution. Yamada *et al.* (1980) postulated that the fading phenomenon of anthocyanins in the presence of AM and CD's was caused by the conversion of the flavylium ion into the pseudobase in two steps:- 1) the formation of an inclusion complex of the anthocyanin with the CD, and 2) conversion of the flavylium ion to the pseudobase by a catalytic action of the CD.

The addition of the KOH/HCl control solution caused an increase in colour in most anthocyanins at pH 4. The increased absorbance is thought to be due to decreased water availability (*i.e.* water activity), and therefore the hydration equilibrium was driven towards the coloured flavylium cation rather than the colourless pseudobase. Goto *et al.* (1976) reported that anthocyanin quinonoidal bases, as well as the flavylium ions were strongly stabilised and did not form colourless pseudobases by hydration when dissolved

in concentrated aqueous solutions of neutral salts such as $MgCl_2$ and NaCl. It has been suggested that colour stabilisation in NaCl may be due to promotion of self-association of anthocyanins whilst the stabilisation of $MgCl_2$ may be due to a reduction in the concentration of free water by hydration of the magnesium ions (Mazza and Miniati, 1993). It is suggested that the increase in colour after the addition of KOH/HCl in these present experiments may be due to one of these reasons.

Recently Chandra *et al.* (1993) reported that the addition of 0.8-1.6% α- and β-CD's to anthocyanins extracted from tart cherries had a "stabilising effect" on the anthocyanins, with β-CD having the largest effect. Juice samples containing these cyclodextrins retained higher levels of anthocyanins after 12 weeks of storage than did solutions without added cyclodextrins. This stabilisation of anthocyanins was thought to occur because the anthocyanin was protected from attack (by water, O_2, furfural, enzymes etc.) by being in the inclusion complex. If this is so, then starch (especially amylose) might also be expected to exhibit a similar protective effect, which could be useful in the long term storage of foods containing anthocyanins. However, Chandra *et al.* (1993) did not mention if there was any colour change (increase or decrease) after the CD's were added, so it is not certain how this result from the work of Chandra *et al.* (1993) relates to the decrease in anthocyanin colour after the addition of CD's which was found here.

The absorbance of anthocyanins (compared with that of the control) increased when glucose, sucrose or maltose were added. This maybe due to a decrease in water activity. Anthocyanin colour is known to increase upon the removal of water by displacement of the hydration-dehydration equilibrium (equation ii and Figure 2.3) towards the coloured species (Brouillard, 1983). It has been found also that sugar molecules are effective at binding water (Coultate, 1989). There was no change in λ_{max} in the reactions of any of the anthocyanins when these sugars were added. Therefore, this increased colour was probably not due to a copigmentation effect because, typically, copigmentation causes a bathochromic shift in the visible λ_{max} of all anthocyanins, as well as the increase in absorption.

The effect of pectin was inconsistent; with Mv and Pg-glycosides it caused a small increase in colour, whereas with Dp-3,5-glu it led to a small decrease in colour. It appears that the effect of carbohydrates on anthocyanin colour may depend on the identity of the aglycone. Asen *et al.* (1972) have reported that pectin may act as a copigment, so further studies into the effect of pectin are required, especially given that jams and a number of other coloured food products contain significant concentrations of pectin.

The effect of carbohydrates (e.g. AM, AP, α-CD, β-CD, glucose, sucrose and maltose) on anthocyanin colour was more dramatic at pH 4 than at pH 2. Although this was due partly to the greatly reduced absorbance of the anthocyanins at pH 4, it is also thought to be caused because at pH 2 the anthocyanins exist in the flavylium form, whereas at pH 4 the anthocyanin becomes hydrated and therefore loses colour.

Therefore, because the pH of plant vacuoles is acidic, it is possible that the presence of carbohydrates in the vacuole may have an effect on the colour shown by the anthocyanins. These results are obviously going to be of importance to the food industry and quality control of food products because, in fruit juices and other food products the colour of anthocyanins could be affected by the levels of starch and sugars, as well as the pH and copigments present. This is especially important in products such as jams, where there are high concentrations of sugars and pectins and the pH is critical, as well as other products such as fruit juices and purees. This phenomenon is potentially important for industries which rely on the natural colour of anthocyanins to produce food products with pleasing appearances.

This work has been published (Lewis *et al.*, 1995) and a copy of this paper is shown in Appendix 5.

CHAPTER 3

Identification and quantitative determination of the anthocyanins, flavonoids and phenolic acids in *Solanum tuberosum* cultivars and other *Solanum* species

3.1 Introduction

The major focus of this project was on the biosynthesis of anthocyanins, so it was necessary to include a study of the precursors (phenolic acids) and related compounds (flavonoids). There has been no comprehensive study of all of these compounds in *S. tuberosum*, although Harborne (1960a and b; 1967) studied anthocyanins and flavonoids, and Wietschel and Reznik (1980a and b) studied flavonoids, in a number of *Solanum* species, and a number of workers have studied phenolic acids in *S. tuberosum* cultivars (Sosulski *et al.*, 1982; Lyon and Barker, 1984; Malmberg and Theander, 1985; Kumar *et al.*, 1991; Ramamurthy *et al.*, 1992; Onyeneho and Hettiarachchy, 1993; de Sotillo *et al.*, 1994a and b). In this chapter, the quantities of phenolic acids, flavonoids and anthocyanins in a number of *S. tuberosum* cultivars with a range of different coloured tubers, and tubers with different intensities of colour were measured. This was carried out to determine if any relationships between the concentrations of these compounds existed, and to provide insight into the biochemical relationships of these compounds. Analysis of the phenolic acids, flavonoids and anthocyanins in flowers and leaves was also carried out, to determine whether or not these organs had similar regulation of anthocyanin and flavonoid biosynthesis as tubers. Enzyme activities from tubers for a number of enzymes involved in the phenylpropanoid and flavonoid pathways were measured, and the activities compared with the concentrations of anthocyanins, flavonoids and phenolic acids.

A number of other *Solanum* species were also analysed to determine if there were any taxonomic differences between these species and *S. tuberosum*, that is, whether there were differences in substitution patterns of the B-ring and/or in glycosylation or acylation patterns, or in quantities of compounds. The analyses of diseased tubers were also included in this chapter. Infection by pathogens and wounding is known to alter the production of phenolics in many plants (Hahlbrock and Scheel, 1989), including potatoes (Kuc *et al.*, 1956; Kuc, 1957; Lee and Le Tourneau, 1958; Friend *et al.*, 1971) It was

Note that throughout this chapter, the term "flavonoid" refers to all the classes of flavonoid compounds except for the anthocyanins, as these are reported and discussed separately from the other flavonoids.

thought that analysis of diseased tubers may provide some insight to the biochemistry of these pathways.

Before any investigation into the biochemistry of phenolics could be carried out, it was necessary to first establish suitable methods for the analysis of these compounds, a large part of which was the identification of the compounds found in potato plants. Therefore, in this chapter the results section is split into two parts. Part one describes the identification of anthocyanins, flavonoids and phenolic acids, in tubers (skin and flesh), flowers and leaves, in *S. tuberosum* and the other *Solanum* species. In part two, the quantitative measurement of these compounds in tubers (skin and flesh), flowers and leaves of thirty cultivars of *S. tuberosum*, and also another eight *Solanum* species is reported, and the effect of disease on the concentrations of these compounds was investigated. The activities of a number of enzymes involved in the phenylpropanoid and flavonoid pathways were measured, and correlated with concentrations of anthocyanins, flavonoids and phenolic acids in the tubers of five *S. tuberosum* cultivars. Both parts of this chapter are discussed together in Section 3.6, the discussion.

3.1.1 Methods of identification of anthocyanins and flavonoids

3.1.1.1 Extraction

Phenolics and flavonoid aglycones (polyphenolics) are slightly acidic, and therefore dissolve most easily in alkaline solvents. However, most flavonoids, especially the anthocyanins, will degrade in alkali, particularly in the presence of O_2 (Markham, 1982). The more polar flavonoids, possessing a number of unsubstituted hydroxyl groups or a sugar, are generally soluble in polar solvents such as ethanol, methanol, acetone, dimethyl sulphoxide and water. The presence of an attached sugar tends to make the flavonoid more water soluble, and combinations of the above solvents with water are better solvents for glycosides. In contrast, less polar aglycones, such as isoflavones, flavanones, and highly methoxylated flavones and flavonols tend to be more soluble in solvents such as ether and chloroform (Markham, 1982).

For the extraction of anthocyanins it is essential to include an acid in the extraction solvent because anthocyanins are only stable under acidic conditions. The most commonly used solvent for anthocyanin and flavonoid extraction is HCl (usually 0.1-1.0%) in methanol or ethanol (Francis, 1989). However, extraction with solvents containing HCl may result in degradation of these compounds, especially when acylated, due to the low pH. To avoid degradation it is advisable to use solvents containing weaker acids such as acetic, tartaric or citric acid (Strack and Wray, 1989).

3.1.1.2 Identification and analysis by chromatographic methods

Early studies of the flavonoids were limited to qualitative analysis using the techniques of paper chromatography (PC), thin-layer chromatography (TLC) or, more recently, column chromatography. There have been many solvent and spray reagents developed to aid in the separation and identification of flavonoids by PC or TLC (Markham, 1982; Grayer, 1989). Comprehensive tables of data detailing Rf values and colours in various spray reagents for all the common and uncommon, flavonoids are available (Mabry *et al.*, 1970; Ribéreau-Gayon, 1972). Whilst these methods are still used and are extremely useful, there is a movement towards the use of high performance liquid chromatography (HPLC) to study flavonoids because it has a number of advantages over other methods of analysis. HPLC can be used for the separation, quantitative determination and identification of flavonoids, and is generally more sensitive than PC or TLC. However, visible detection systems used for HPLC analyses of anthocyanins can be insensitive, whilst TLC derivatisation techniques with spray reagents can be very sensitive. Reversed phase (RP) C_{18}-columns (containing silica derivatised with octadecyl trichlorosilane) are the most commonly used for flavonoid analysis, with methanol/water or acetonitrile/water the preferred solvent systems. Onyeneho and Hettiarachchy (1993) used a RP C_{18}-column with methanol, acetonitrile, and a pH 5.4 buffer to separate and identify the phenolic acids present in potato peel extracts.

3.1.1.3 Identification and analysis by spectral methods

UV/visible spectroscopy

UV and visible absorption spectroscopy is useful in the identification of flavonoids and anthocyanins because each class of compound (anthocyanin, flavonol, flavone, flavanone, etc.) has a characteristic absorption spectrum. An excellent range of spectral data is presented in Mabry *et al.* (1970).

The use of spectral shift reagents

By measuring the spectrum in methanol, together with the bathochromic or hypsochromic shift caused by the addition of shift reagents, it is possible to identify many anthocyanins and flavonoids (Mabry *et al.*, 1970; Markham, 1982).

Methanolic spectrum. The methanolic spectrum of flavonoids shows two major absorption peaks between 240 and 550nm. These two peaks are commonly referred to as Band I (usually 300 to 550nm) and Band II (usually 240 to 280nm). Band I is associated with absorption due to the B-ring, and Band II with absorption involving the A-ring. Therefore, the intensity and ratio of Band I and II are altered by the oxygenation

pattern on the A- and B-rings (Mabry *et al.*, 1970; Markham, 1982). The class of flavonoid may be deduced from its spectrum in methanol, as shown in Table 3.1.

Table 3.1 UV/visible absorption ranges for flavonoids in methanol (from Markham, 1982).

Flavonoid type	Band II (λ_{max})	Band I (λ_{max})
Flavone	250-280	310-350
Flavonols (3-hydroxyl substituted)	250-280	330-360
Flavonols (3-hydroxyl free)	250-280	350-385
Isoflavones	245-275	310-330 shoulder
Isoflavones (5-deoxy-6,7-dioxygenated)	-	320
Flavanones and dihydroflavonols	275-295	300-330 shoulder
Chalcones	230-270 (low intensity)	340-390
Aurones	230-270 (low intensity)	380-430
Anthocyanidins and anthocyanins	270-280	465-560

Sodium methoxide spectrum. With the addition of sodium methoxide (NaOMe) or a strong base (e.g. NaOH) to the sample in methanol, all the hydroxyl groups on the flavonoid nucleus are deprotonated. This spectrum gives an indication of the hydroxylation pattern, and degradation of the spectrum with time indicates alkali sensitive groups.

Sodium acetate spectrum. Sodium acetate (NaOAc) is a weaker base than sodium methoxide, and only deprotonates the more acidic hydroxyl groups. Its chief use is to detect the presence of 7- and 4'-hydroxyl groups.

Sodium acetate/boric acid spectrum. In the presence of sodium acetate, boric acid (H_3BO_3) chelates with *o*-dihydroxyl groups at all locations on the flavonoid nucleus, except C-5,6, and is used to detect the presence of *o*-dihydroxyl groups.

$AlCl_3$ and $AlCl_3$/HCl spectra. Aluminium chloride ($AlCl_3$) forms acid-stable complexes between hydroxyls and neighbouring ketones, and acid-labile complexes with *o*-dihydroxyl groups, so these reagents can be used to detect both groupings. The $AlCl_3$ spectrum represents the effects of both these complexes on the spectrum, whilst the $AlCl_3$/HCl spectrum represents the effect of only the hydroxy-keto complexes (Mabry *et al.*, 1970; Markham, 1982).

Spectroscopy is also useful for the identification of anthocyanins, where the addition of $AlCl_3$ aids the detection of unsubstituted *o*-dihydroxyl groups. The identity of substituted acyl groups may be determined by the λ_{max} position of the acyl group (300-330nm). The number of acyl groups is indicated by the ratio of the acyl group λ_{max} and the visible $\lambda_{max}$of the pigment (500-540nm). The ratio $A_{440}/A_{\lambda max}$ gives an indication of whether the 5-hydroxyl group is free or substituted (Ribéreau-Gayon, 1972).

Mass spectroscopy

Mass spectroscopy (MS) may be performed on sub-milligram quantities of compound, and can be used to determine the molecular weights and chemical formulae of the flavonoid. Information on the substitution patterns of the A- and B-rings may also be obtained. Electron impact MS, a method which is commonly used for a number of other classes of compounds, can only be used on those flavonoid aglycones that are volatile under the probe conditions. Because flavonoid glycosides are usually non-volatile, other MS techniques, such as fast atom bombardment (FAB) or field desorption MS, must be used (Grayer, 1989). FAB-MS is the most common ionisation method for flavonoids because it provides an ideal technique for the analysis of highly polar and ionic classes of compounds, without having to derivatise them. It also has the advantage of giving a molecular ion and various fragmentation ions that give direct information on the structure of the flavonoid (Strack and Wray, 1989).

Nuclear magnetic resonance spectroscopy

Both proton (^1H) and carbon-13 (^{13}C) nuclear magnetic resonance (nmr) spectroscopy are useful for the identification of flavonoids.

^1H-nmr can be used to :-

1) determine the oxygenation pattern of the flavonoid,
2) establish the number of sugars present in glycosides, and
3) distinguish between the different classes of flavonoids.

^{13}C-nmr gives information about :-

1) the class of flavonoid,
2) the carbon skeleton of the flavonoid, for example, the number of carbon atoms and which of these carbons is oxygenated,
3) the identification of the sugar(s) in flavonoid glycosides,
4) the presence and identity of any acyl groups, and
5) the position of linkage between the flavonoid aglycone, sugars and acyl groups.

The disadvantage of ^{13}C-nmr is that it requires a relatively large (15mg or more) sample (Grayer, 1989).

3.1.2 Anthocyanins, flavonoids and phenolic acids found in Solanaceae, especially *Solanum* species

3.1.2.1 Anthocyanins

The first detailed examination of the anthocyanins present in the cultivated potato (*S. tuberosum*) was carried out by Chmielewska (1936) who studied the pigments contained in the tuber skin and flesh of a purple-black cultivar called "Negresse" (also later called "Congo"). Thus, the first anthocyanin to be chemically identified from potatoes was negretein [malvidin 3-(*p*-coumaroyl-rutinoside)-5-glucoside] (Figure 3.1).

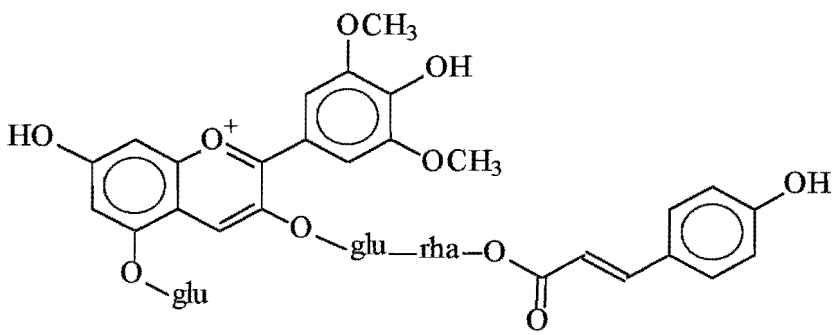


Figure 3.1 Structure of Negretein [malvidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside].

From his work on the tubers and flowers of various coloured cultivars, Harborne (1960a) described the isolation and characterisation of ten anthocyanins from diploid and tetraploid potato species. Depending on the colour, the 3-rutinoside-5-glucoside (3-rut-5-glu) derivatives of all the six common anthocyanins, acylated with *p*-coumaric acid, plus the 3-rutinoside (3-rut) derivatives of pelargonidin (Pg), cyanidin (Cy), delphinidin (Dp) and petunidin (Pt) were found. The 3-rut-5-glu derivatives of all six common anthocyanins have been commonly found in an acylated form in many plants of the Solanaceae (e.g. potato, tomato, petunia, aubergine) (Harborne, 1967).

In a survey of pink and purple pigmented *S. tuberosum* tubers and sprouts, Howard *et al.* (1970) found that single anthocyanidins were typical of *S. tuberosum* cultivars, rather than the mixtures commonly found in cultivated forms of diploid potatoes. However, Sachse (1973) found that Desirée contained both Pg and peonidin (Pn) glycosides. It is possible that the PC procedures used by Howard *et al.* (1970) could only detect the major anthocyanins and not the more minor anthocyanins.

3.1.2.2 Other flavonoids and phenolic acids

Chlorogenic acid was one of the first phenolics to be studied in potatoes. Chlorogenic acid and caffeic acid were found in potato peel by Kuc *et al.* (1956) and Kuc (1957), and Lee and Le Tourneau (1958) found that potato leaves contained high concentrations of chlorogenic acid, whereas roots and stems contained very little. Chlorogenic acid biosynthesis and induction by light and wounding in tubers was studied in detail by Zucker (1963; 1965) and Lamb and Rubery (1976).

In a study of phenolics from over seventy species of South American and Mexican tuberous *Solanum* plants, Harborne (1960b) found that the flowers and leaves of *S. pinnatisectum* contained scopolin, cichorin, aesculin, chlorogenic acid, rutin, isoquercitrin, plus another seven minor phenolics. However, the tubers contained only chlorogenic acid and scopolin (plus anthocyanins in the coloured varieties). Only two other species of tuberous *Solanum* plants, *S. jamesii* and *S. sambucinum* contained coumarins in their flowers. They contained the same major polyphenols as *S. pinnatisectum* except that in *S. jamesii* aesculin was absent but a *p*-coumaric acid ester was present. No coumarins were found in flowers of domestic varieties of the cultivated potato (Harborne, 1960b).

The flavonoids occurring in the *Solanaceae* have been studied thoroughly. Harborne (1960a) isolated the flavonols, kaempferol (Km), quercetin (Qu) and myricetin (My), from the tubers and flowers of various coloured cultivars of diploid and tetraploid potato species. The pattern of leaf flavonoids in tuberous *Solanaceae* was investigated by Wietschel and Reznik (1980a and b) in a comprehensive study involving 107 species. Thirty eight different flavonol-glycosides and one flavone-glycoside were characterised. These included: Qu-3-arabinoside (Qu-3-ara), Qu-3-xyloside (Qu-3-xyl), Qu-3-galactoside (Qu-3-gal), Qu-3-neohesperidoside and Qu-3-(2^G-rhamnosyl-rutinoside) (Qu-3-(2^G-rha-rut); Km-3-gal, Km-3-neohesperidoside, Km-3-(2^G-rha-rut) and Km-3-sophorotrioside-7-rha; and isorhamnetin-3-gal and isorhamnetin-3-rut; all acylated with *p*-coumaric or ferulic acid (Wietschel and Reznik, 1980a). Qu-derivatives were the predominating glycosides, accompanied by Km, and in some cases isorhamnetin and My-glycosides, but in much smaller quantities (Wietschel and Reznik, 1980b). Some of these flavonoids have been found in *S. tuberosum* and Harborne (1967) reported that Km-3-(2^G-glu-rut) and Qu-3-(2^G-glu-rut) were found from *S. tuberosum* petals and Km-3-sophoroside-7-rha and Km-3-sophorotrioside-7-rha were found from *S. tuberosum* seeds.

In 1982, Sosulski *et al.* investigated the phenolic content of potato flour (from peeled, freeze-dried tubers of cv. Netted Gem) reporting that it contained high concentrations of chlorogenic acid and only traces of other phenolic acids, except for caffeic acid (a component of chlorogenic acid) (Table 3.2).

Onyeneho and Hettiarachchy (1993) studied the phenolic acid composition of potato peels and found that the phenolic acid composition of different cultivars was unrelated to the colour (*i.e.* anthocyanin content) of the peels. Protocatechuic acid, chlorogenic acid and caffeic acid were the major phenolic acids in all peels (Table 3.2), whilst red peel contained a higher percentage of polyphenols, such as anthocyanins.

Table 3.2 Phenolic acids found in potatoes.

Phenolic acid	ppm*	Amount (mg per 100g extract)**	
		Viking (red)	Kennebec (brown)
chlorogenic acid	341.3	753.0	821.3
caffeic acid	60.0	278.0	296.0
p-coumaric acid	5.1	45.6	41.8
ferulic acid	4.8	174.0	192.0
gallic acid	ND	58.6	63.0
p-hydroxybenzoic acid	trace	87.0	82.0
vanillic acid	trace	48.0	43.0
quinic acid	trace	ND	ND
sinapic acid	-	ND	ND
protocatechuic acid	-	216.0	256.0
syringic acid	-	ND	ND
p-(hydroxyphenyl)-acetic acid	-	ND	ND

ND = not determined

- = absent

* from Sosulski *et al.* (1982)

** from Onyeneho and Hettiarachchy (1993)

Table 3.3 Classification of potato species (adapted from Hawkes, 1990).

Genus: *Solanum* L.			
Subgenus: *Potatoe*			
Section: *Petota*			
Subsection: *Estolonifera*			
Series I-II			
Subsection: *Potatoe*			
Super-series: *Stellata*			
Series I-IX			
Super-series: *Rotata*			
Series X:	*Megistacroloba*		
		S. sanctae-rosae	
Series XI:	*Cuneoalata*		
Series XII:	*Conicibaccata*		
Series XIII:	*Piurana*		
Series XIV:	*Ingifolia*		
Series XV:	*Maglia*		
Series XVI:	*Tuberosa*		
	Wild species:	Group (i) Mex., Ven., Col. & Equ.	
		Group (ii) Peru:	
		S. sparsipilum	
		Group (iii) Bol., Arg. & Chlie:	
		S. berthaultii	
		S. gourlayi	
		S. oplocense	
		S. sparsipilum	
		S. speggazzinii	
	Cultivated species:	*S. stenotomum*	
		S. tuberosum	
Series XVII:	*Acaulia*		
		S. acaule	
Series XVIII:	*Longipedicellata*		
Series XIX:	*Demissa*		

Abbreviations

Arg.	Argentina	Equ.	Equador
Bol.	Bolivia	Mex.	Mexico
Col.	Colombia	Ven.	Venezuela

3.1.3 Potato systematics

A brief summary of potato taxonomy is included here, because a number of *Solanum* species were investigated in this work. There are a large number of species in the genus *Solanum* (approximately 900), and taxonomists have established various subgenera, of which the subgenus *Potatoe* includes the potatoes and various other groups of species which are taxonomically related to them in some way (Hawkes, 1990). The subgenus *Potatoe* has been further divided into sections, of which the section *Petota* includes the tuber-bearing species. Within this section (*Petota*) there are two subsections, *Estolonifera* and *Potatoe* (Table 3.3). Hawkes (1989) added two super-series in the *Potatoe* subsection (*Stellata* and *Rotata*) to differentiate between species with stellate (star-shaped) or rotate (round) corollas. Below the rank of the super-series are the series, two in subsection *Estolonifera*, nine in the super-series *Stellata*, and ten in the super-series *Rotata* (Table 3.3).

The extensive geographical distribution of wild potatoes indicates a wide range of ecological diversity and a range of adaptations to extremes of temperature and humidity far surpassing that of the cultivated species. Thus, some species such as *S. acaule* can withstand sub-zero temperatures whilst others, such as *S. berthaultii,* are adapted to hot, dry semi-desert conditions. Apart from the lowland tropical rain forest, wild potato species appear to have penetrated into nearly every natural habitat, as well as a number of man-made habitats; thus many, such as *S. sparsipilum,* are commonly found wild in cultivated fields. In addition to the physiological diversity of wild potatoes from an ecological and phytogeographical point of view, wild potatoes exhibit a wide diversity of resistance to fungal, bacterial and viral diseases, as well as to insect, arachnid and nematode pests. Some also possess high dry matter content in tubers, though most have watery tubers that often contain unacceptably high alkaloid contents. This morphological diversity has potential economic importance in providing useful characters of interest for potato breeders (Hawkes, 1990). Therefore, it was of interest to identify and quantify the phenolic acids, flavonoids and anthocyanins in a number of other *Solanum* species, and to determine if there was any significant difference between these species and *S. tuberosum.*

3.1.3.1 Solanum species used for analyses

Nine species of *Solanum* were available for use in the present study to compare concentrations of phenolics: these were *S. acaule, S. berthaultii, S. gourlayi, S. oplocense, S. sanctae-rosae, S. sparsipilum, S. speggazzinii, S. stenotomum* and *S. tuberosum.* Their classification is shown in Table 3.3.

***S. acaule* Bitt.** is a tetraploid species found in southern Peru, Bolivia, and northern Argentina, and is tolerant of frost, heat and drought. This species is divided into three geographical subspecies, subsp. *acaule*, subsp. *aemulans* and subsp. *punae*. *S. acaule* subsp. *acaule* Bitt. is found at altitudes of 3700-4200m, but sometimes up to 4650m in alpine meadows by paths, walls, ditches and arable fields. *S. acaule* subsp. *aemulans* Bitt. et Wittm. is found only in Argentina at 2950-3500m in open stony places and alpine meadows (Hawkes, 1990).

***S. berthaultii* Hawkes** is a diploid species found throughout Bolivia at an altitude of 2400-2750m in dry inter-andine valleys among scrub and cactus vegetation (Hawkes, 1990).

***S. gourlayi* Hawkes** is found in central to southern Bolivia and north-western Argentina. There are four subspecies. *S. gourlayi* subsp. *gourlayi* Hawkes is only found in Jujuy, Argentina at 1900-3600m in dry vegetation amongst grasses, cacti and small shrubs. Diploid and tetraploid cytotypes occur (Hawkes, 1990).

***S. oplocense* Hawkes** is found in Bolivia and Argentina at altitudes of 2600-3500m in dry thorn-bush scrub, hedges, cultivated fields and cactus vegetation. It is the only species known with diploid, tetraploid and hexaploid cytotypes. The diploid cytotype is very rare, but the tetraploid and hexaploid are common (Hawkes, 1990).

***S. sanctae-rosae* Hawkes** is a frost-tolerant diploid species found in north-western Argentina in high mountain pastures and sandy and rocky places from 2500-3800m (Hawkes, 1990).

***S. sparsipilum* Bitt.** is a very polymorphic weed and is found in Peru and Bolivia at 2800-3800m in fields, walls and field-borders. It is very similar to *S. tuberosum* subsp. *andigena* and is believed to have been one of its diploid prototypes. It is unique in that it has two quite distinct distribution areas, Group (ii) in Peru and Group (iii) in Bolivia (Table 3.3), however it is thought to have originated in Bolivia (Hawkes, 1990).

***S. speggazzinii* Bitt.** is a diploid species which is found in Argentina at 1900-3100m in dry inter-andine valleys under the shade of trees and bushes and also in fields and field-borders (Hawkes, 1990).

***S. stenotomum* subsp. *stenotomum* Juz. et Buk.** is a diploid found in southern Peru and northern Bolivia at high altitudes. It is a highly variable species which is possibly ancestral to all the other cultivated potatoes (including *S. tuberosum*) (Hawkes, 1990).

***S. tuberosum* L.** is a tetraploid species with two recognised subspecies, subsp. *tuberosum* and subsp. *andigena*:-

a) Subsp. *tuberosum* forms tubers under long days, or under short days in the tropics at lower altitudes only (500-2000m). This subspecies was derived from subsp. *andigena* probably on two separate occasions; first in Chile where subsp. *andigena* was carried by Indian tribes migrating southwards from the Bolivian Andes; and secondly subsp. *andigena* was brought to Europe after the Spanish conquest where, under similar climatic and daylength conditions to those in Chile, the subsp. *tuberosum* was formed again, partly as a result of artificial selection. It was originally only found on the coastal regions of central Chile and is still widely grown in these regions at low altitudes, but is also now found world wide (Hawkes, 1990).

b) Subsp. *andigena* forms tubers at high altitudes only (over 2000m) under short day conditions. This subspecies originated either partly or wholly from *S. stenotomum* in the Andes of Peru and Bolivia. It is found in the Andes of the following countries; Venezuela, Colombia, Equador, Peru, Bolivia, north-western Argentina, and also sparingly in Guatemala and Mexico (Hawkes, 1990).

3.2 Materials

3.2.1 Flavonoid standards

Cyanidin (Cy), cyanidin-3-galactoside (Cy-3-gal), cyanidin-3-glucoside (Cy-3-glu), cyanidin-3-rhamnosyl-glucoside (Cy-3-rha-glu), cyanidin-3-rutinoside (Cy-3-rut), delphinidin (Dp), delphinidin-3-rhamnosyl-glucoside (Dp-3-rha-glu), delphinidin-3-rutinoside (Dp-3-rut), kaempferol-3-rutinoside (Km-3-rut), malvidin (Mv), myricetin (My), pelargonidin (Pg), pelargonidin-3-rhamnosyl-glucoside (Pg-3-rha-glu), pelargonidin-3-rutinoside (Pg-3-rut), peonidin (Pn), petunidin (Pt), quercetin-3-galactoside (Qu-3-gal), quercetin-3-glucoside (Qu-3-glu), quercetin-3-rhamnoside (Qu-3-rha) and quercetin-3-xyloside (Qu-3-xyl) were obtained from Plantech (UK). Catechin, epicatechin, kaempferol (Km), rutin and sinapic acid were obtained from Fluka (Switzerland). Apigenin, eriodictyol and luteolin were obtained from Roth AG (Germany). Quercetin-3-arabinoside (Qu-3-ara) was obtained from Extrasynthase (France). Procyanidin B2 and procyanidin B5 were kindly donated by Dr L. Porter (Industrial Research Limited, Gracefield Research Centre, Lower Hutt, NZ). Other flavonoid and phenolic acid standards were obtained from Sigma (USA).

3.2.2 Enzyme assays

Materials used in enzyme assays are listed in Section 5.2.1.

3.2.3 TLC developing solvents

15% HOAc:- 15% (v/v) acetic acid in water
BAW:- *n*-butanol : acetic acid : water (4 : 1 : 2.2)
BBPW:- *n*-butanol : benzene : pyridine : water (5 : 1 : 3 : 3; upper phase)
Forestal:- acetic acid : HCl : water (30 : 3 : 10)

3.2.4 TLC spray reagents

Aniline phthalate:- aniline (0.92ml) and phthalic acid (1.6g) were dissolved in 100ml H_2O saturated *n*-butanol.
NA-reagent (Naturstoffreagenz A or diphenyl-boric acid-ethanolamine complex):- 1% (w/v) solution in methanol.
Sodium carbonate:- 1% (w/v) aqueous solution.

3.2.5 Shift reagents

NaOH:- 2M aqueous solution.
$AlCl_3$:- five grams of fresh, anhydrous $AlCl_3$ was added to 100ml methanol (made fresh as required).
HCl:- concentrated HCl (50ml) was added to 100ml water.
H_3BO_3:- ground to a fine powder in a mortar and pestle and added as a solid.
NaOAc:- ground to a fine powder in a mortar and pestle and added as a solid.

3.3 Methods

3.3.1 Samples

Skin:- skin samples were taken from around the centre of the tuber (i.e. between the stem and bud ends) . Attempts were made to use only the outer coloured layer(s) of cells.

Flesh:- flesh samples were taken from the centre portion of the tuber (i.e. between the stem and bud ends) and all skin was removed.

Skin and flesh samples that were not analysed immediately were frozen in liquid N_2 and stored at -80°C.

Flowers:- flowers were picked, the petals removed and freeze-dried immediately.

Leaves:- leaf material was freeze-dried immediately after picking.

Freeze-dried material was stored in a dark, dry cupboard at room temperature until required for analysis.

3.3.2 Extraction of anthocyanins and flavonoids

Samples (0.5g) were ground to a fine powder in a mortar and pestle using liquid N_2 then extracted four times with 15% (v/v) acetic acid in methanol over a period of 30min. The combined extracts were centrifuged at 12 000g for 10min at 4°C and the supernatant was evaporated to dryness at 40°C and redissolved in 1.5ml of extraction solvent. For analysis by HPLC this final extract was centrifuged at 16 000g for 20min at 4°C and the supernatant stored at -20°C.

3.3.3 Identification of anthocyanins and flavonoids

3.3.3.1 Thin-layer chromatography

Preliminary analysis of the flavonoids was carried out by two-dimensional (2-D) TLC on plastic-backed cellulose plates (Merck No 5577), developed with BAW followed by 15% acetic acid. The dry TLC plates were observed under UV light and then exposed to NH_3 fumes under UV light or sprayed with one of the following spray reagents in order to visualise spots and characterise groups of compounds.

1) NA-reagent:- 3',4'-dihydroxyflavonols appear orange and 4'-hydroxyflavonols produce yellow-orange spots (Markham, 1982).

2) Sodium carbonate:- under UV light, phloretin appears violet and flavonols produce yellow, orange or brown spots (Gage *et al.*, 1951).

Tentative identifications were made by calculation of Rf values and comparison with known standards and published data (Harborne, 1959; 1967; Mabry *et al.*, 1970). The Rf values presented in this thesis will be expressed as a percentage, rather than as a proportion.

3.3.3.2 HPLC

Analytical HPLC

Analytical HPLC was carried out with the assistance of Dr K. Sutton at the NZ Institute for Crop & Food Research Ltd, Lincoln. A Waters 600 solvent delivery/control system with a Waters WISP 712 automatic sample injector and a Waters 490 variable wavelength UV/visible detector were used to separate, identify and quantify the anthocyanins, flavonoids and phenolic acids. The column used was a 220 x 4.6mm Applied Biosystems Brownlee Aquapore RP-18 column fitted with a 15 x 3.2mm RP-18 guard column. Chromatographic traces were recorded using the Waters Dynamic Solutions program "Maxima". Solvents used for elution were: A) 10% (v/v) acetic acid in water and B) acetonitrile. The solvents were vacuum filtered through a 0.22μm filter. Degassing was achieved by rapid sparging with helium (100ml/min for 10min), followed by constant slow bubbling of helium into capped, vented solvent reservoirs (5ml/min). Samples (5-20μl) were injected onto the column, which was maintained at 30°C using a Waters column heater. A flow rate of 1ml/min was used with a linear 30min solvent gradient from 0 to 30% B followed by a 5min hold at 30% B. The column was washed with 50% B for 5min, then returned to 0% B and re-equilibrated for 10min before the next analysis. The eluted components were monitored at 280nm for catechins, flavanones, dihydroflavonols and some phenolic acids, 313nm for the remaining phenolic acids, 350nm for flavonols and flavones, and 530nm for anthocyanins. The individual compounds were identified and quantified by comparison of retention times of standard solutions of known concentration (where available). The identification was confirmed by spectral analyses and in some cases TLC. Where standards were not available, quantification was based on an average value for that class of compound, because responses were similar within classes.

Diode-array analytical HPLC

The method was carried out as above except that a Waters "Discovery" System was used. This consisted of the Waters 625 quaternary gradient pump/controller, Waters 717 autosampler and the Waters 996 photodiode array detector (scanning from 200-600nm). The software used was Waters Millenium (Version 2.1) running under Windows 3.1.

Preparative HPLC

Preparative HPLC was used to isolate individual anthocyanins and flavonoids. Samples (10-20g) were extracted as above (Section 3.3.2), and then cleaned and semi-purified by a RP-18 bench column before HPLC. Preparative HPLC work was carried out using a Varian 5000 Vista Series Liquid Chromatograph with a 300 x 25mm Rainin Microsorb RP-18 column (pore size 60Å) with a 110 x 25mm guard column. The solvents, A and B, were the same as for analytical HPLC. Samples of up to 400µl were injected on to the column, and a flow rate of 12ml/min was used. Tuber anthocyanins were detected at 530nm with a linear 20min solvent gradient from 8 to 10% B, followed by a linear gradient from 10 to 20% B for the following 15min. Flower flavonoids were detected at 350nm with a linear 30min solvent gradient from 0 to 30% B. In both cases the column was washed with 100% B for 5min, then returned to the starting concentration and re-equilibrated. The collected fractions, usually each containing a single peak, were concentrated *in vacuo*. These compounds were further identified by TLC, acid hydrolysis, MS, and/or UV/visible spectroscopy, including the use of shift reagents.

3.3.3.3 Acid hydrolysis of glycosides

To elucidate further the structures of the anthocyanins and flavonoids separated by preparative HPLC, and also to determine the identity of the aglycones present in these extracts, the glycosides were hydrolysed in acid to cleave the sugar from the aglycone. The anthocyanin or flavonoid (in an Eppendorf tube) was dissolved in methanol and an equal volume of 4M-HCl was added. The solution was heated at 90°C for 2-24h until hydrolysis was complete, as determined by TLC developed in BAW. To isolate the sugars and the aglycone for further analysis, the methanol was removed from the solution *in vacuo*, then the aqueous solution extracted twice with an equal volume of ethyl acetate. The aqueous and ethyl acetate phases were separated, with the aglycone present in the upper ethyl acetate fraction and the sugars (and any remaining unhydrolysed glycosides) remaining in the aqueous fraction (Markham, 1982).

The aglycones were identified by :-

1) TLC using the solvents: Forestal, 15% acetic acid or BAW; calculation of Rf values and comparison with standards, and
2) UV/visible spectroscopy and comparison with standards and published spectra (Mabry *et al.*, 1970).

Sugars were identified by TLC with the solvent system BBPW. The plates were sprayed with aniline phthalate and heated in an oven at 90°C until brown spots appeared (5-10min). Rf values of sample sugars were compared with known sugar standards.

3.3.3.4 UV/visible spectroscopy

Anthocyanins

The spectrum of each anthocyanin sample in 15% (v/v) acetic acid in methanol from 260 to 700nm was recorded, and the λ_{max} in the visible region used to give an indication of the aglycone identity. To estimate the number and identity of acyl substituents present the ratio of λ_{max} of the acyl group (300-330nm) compared with the visible λ_{max} (500-540nm) was calculated. The $A_{440}/A_{\lambda max}$ was calculated to determine 5-hydroxyl substitution patterns (Ribéreau-Gayon, 1972).

Flavonoids

The spectra of flavonoid samples in methanol (500µl) were recorded from 220 to 580nm, and then re-recorded after addition of various spectral shift reagents.

1) Three drops of 2M-NaOH were added to the flavonoid sample in methanol. The spectrum was recorded immediately, and then again after 5min.

2) To another sample in methanol
   a) three drops of $AlCl_3$ were added and the spectrum recorded, followed by,
   b) the addition of two drops of HCl and the spectrum re-recorded .

The bathochromic shifts after the addition of $AlCl_3$ and HCl were compared.

3) To a third sample in methanol
   a) NaOAc powder was added (until 2mm coated the bottom of the cuvette), mixed thoroughly, and the spectrum recorded immediately, and then again after 5min, followed by,
   b) the addition of H_3BO_3 powder (until 1mm coated the bottom of the cuvette), mixed thoroughly, and the spectrum re-recorded.

Sample spectra and shifts were compared with published data (Mabry *et al.*, 1970).

3.3.4 Enzyme assays

Extraction and assay of enzyme activities were carried out as in Section 5.2.2.

3.3.5 *Solanum tuberosum* cultivars

A range of *S. tuberosum* cultivars were selected to provide a wide variety of tuber colours (Appendix 1, Tables A1.1 and A1.2). They were field grown by staff from the NZ Institute for Crop & Food Research Ltd, Lincoln, in plots at Springs Rd, Lincoln in the 1992/93 and 1993/94 seasons. For the 1992/93 season, flowers, leaves and tubers were harvested, and in the 1993/94 season, only tubers were harvested. Plant material was extracted and analysed by visible spectroscopy, TLC and analytical HPLC, as in Section 3.3.3. To reduce the number of samples to be analysed by HPLC, duplicate samples were not analysed; however, for each skin and flesh sample, tissue was combined from 3-5 tubers, for extracts from flowers, petals were combined from the flowers of as many plants as possible (usually 10-15 plants and a total of about 50 flowers), and for leaves, the 3-4 youngest, fully expanded leaves from each plant, were taken from 5-7 plants, and combined before extraction.

3.3.6 Other *Solanum* species

Seed samples of eight species of *Solanum* were imported from the Scottish Crop Research Institute, Dundee, Scotland. This was the only source of these species in New Zealand and plants were retained in quarantine (NZ Institute for Crop & Food Research Ltd, Lincoln) throughout all growth and extraction processes. Information relating to the species and accessions is shown in Table 3.4.

Approximately 25 seeds from each line were planted on 24 November 1992 by staff at the NZ Institute for Crop & Food Research Ltd, Lincoln, and those that germinated were planted out into 90mm pots in the quarantine glasshouse (Appendix 1, Table A1.3 shows percentage germination). After three weeks the established plants were transplanted into PB8 plastic bags. These were spaced on benches in the quarantine glasshouse with demand watering. Plants were trained up 1.5m poles and stolons were removed to avoid cross-contamination. Flowers and leaves were removed from 2-3 plants from each line, selected to give a range of flower colours (Appendix 1, Table A1.3), and material was frozen (-20°C) until use. Plants began to die in May 1993 and tubers were harvested from the same plants as the flower and leaf samples. Tissue was extracted (as in Section 3.3.2) in the quarantine unit, and analysed by visible spectroscopy and analytical HPLC (as in Section 3.3.3). The waste plant material was autoclaved before disposal.

Table 3.4 Imported *Solanum* species - accession information (from Wilkinson *et al.*, 1994).

Species	Subspecies	Species Code	CPC No	Source	Country	Province	Plant No
S. acaule	*acaule*	ACL	2113	CAR	BOL	Potosoi	1, 7, 11
S. acaule	*aemulans*	ACLAEM	3734	HAW 3396	ARG	La Rioja	2, 4
S. berthaultii	-	BER	4036	CPC 283 x 2731	BOL x ARG	-	1, 2
S. gourlayi	*gourlayi*	GRL	2480	HJE 365	ARG	Salta	6, 9
S. oplocense	-	OPL	3777	HAW 3907	ARG	Jujuy	4, 8
S. sanctae-rosae	-	SCT	3269	DOP s.n.	ARG	-	2, 17
S. sanctae-rosae	-	SCT	3779	HAW 3939	ARG	Salta	4, 6, 8
S. sparsipilum	-	SPL	3488	CPC 62 x 122	BOL	-	2, 6
S. sparsipilum	-	SPL	3563	DOS 3	BOL	Cochabamba	2, 3
S. speggazzinii	-	SPG	3745	HAW 3525	ARG	Catamarca	6, 7
S. speggazzinii	-	SPG	3791	HAW 3425	ARG	La Rioja	2, 15
S. stenotomum	*stenotomum*	STN	4711	CPC 1646 x2464	BOL x ARG	-	5, 12

CPC No : CPC identification number

Source No : collector and number or collection from which the material was originally received

Country : country of origin of the accession

Province : state, province or department from which the accession originated

Plant No : the number of the plant which was analysed - given by NZ Institute for Crop & Food Research Ltd, Lincoln

Abbreviations

ARG : Argentina

BOL : Bolivia

CAR : Cardenas, M., Universidad Mayor de San Simon, Cochabamba, Bolivia

CPC : Commonwealth Potato Collection

DOP : Dodds, K.S., Paxman, G.J., John Innes Institute Expedition 1959-1960

DOS : Dodds, K.S., Simmonds, N.W., John Innes Institute Expedition 1962

HAW : Hawkes, J.G., University of Birmingham, Birmingham, UK

HJE : Hjerting, J.P., Copenhagen, Denmark.

CHAPTER 3 - Part 1. Identification of phenolics

3.4 Results - Part 1

3.4.1 Identification of anthocyanins from tubers

Initial investigation of *S. tuberosum* tuber anthocyanins was confined to extracts from two red-skinned cultivars, Desirée and Red Flesh, and two purple-skinned cultivars, Arran Victory and Urenika. A combined 2-D TLC chromatogram of Desirée and Urenika anthocyanins is shown in Figure 3.2. Three major anthocyanins were separated for each of these cultivars: from Desirée - D1, D2 and D3; and from Urenika - U1, U2 and U3. The results from the 2-D TLC's of the four cultivars studied are summarised in Table 3.5, along with the λ_{max} values which were recorded for each of these extracts.

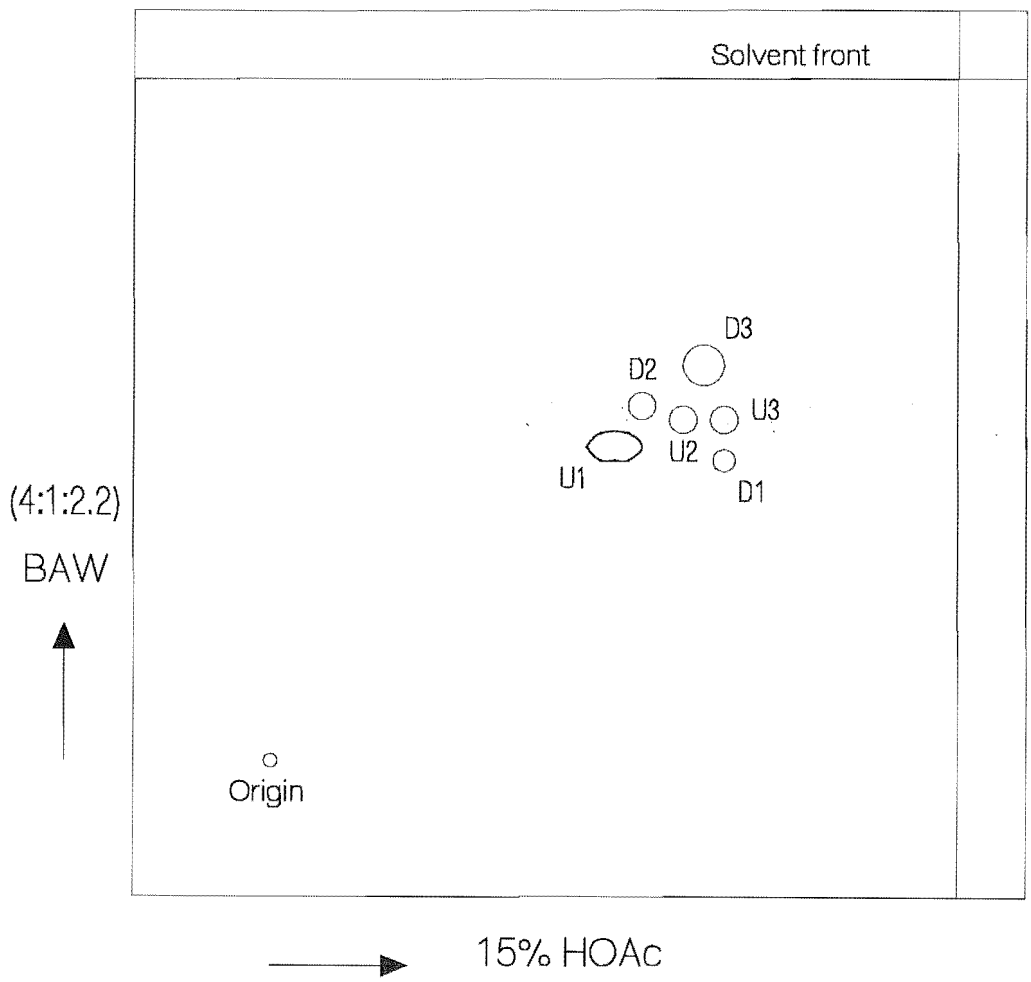


Figure 3.2 Two-dimensional TLC of anthocyanins from Desirée and Urenika tubers. This is a combined TLC which shows anthocyanins D1, D2 and D3 from Desirée and U1, U2 and U3 from Urenika.

Table 3.5 Chromatographic properties and λ_{max} values of tuber anthocyanins from TLC.

Cultivar	Spot no	Rf (a)	Rf (b)	Visible and UV colour	UV + NH_3 colour	λ_{max} (nm)	Tentative identification
Desirée	D1	45.4	66.4	pink	purple	512	Pg-3-rut
	D2	53.7	57.1	pink/purple	purple		Pn-gly
	D3	58.4	64.8	pink	purple		anth-3
Urenika	U1	45.7	51.9	blue/purple	blue/purple	538	anth-1
	U2	49.1	56.9	red/purple	blue		-
	U3	53.2	58.1	red/purple	blue		anth-2
Arran Victory	AV1	43.7	47.0	blue/purple	blue	538	anth-4
Red Flesh	RF1	44.4	66.9	pink	purple	510	Pg-3-rut
	RF2	56.3	64.3	pink	purple		anth-3

(a) Rf (x100) BAW

(b) Rf (x100) 15% acetic acid

Analytical HPLC analysis was in general agreement with the TLC results. Anthocyanins were eluted from the HPLC column with retention times between 17 and 28min. A typical anthocyanin HPLC chromatogram from the purple/black coloured Urenika tubers is presented in Figure 3.3a and shows four anthocyanins. The retention time and λ_{max} of peak 'A' corresponded to that of Dp-3-rut, whereas peak 'B' was thought to be Mv-3-rut. The retention times of the two major peaks found from Urenika tubers (anth-1 eluting at 25.4min and anth-2 eluting at 27.3min) (Figure 3.3a, Table 3.6) did not correspond to those of any available standards, and could not be identified at this stage.

HPLC analysis of Red Flesh anthocyanins revealed a number of anthocyanins present (Figure 3.3b), and this chromatogram was typical of extracts from all red-skinned tubers. All peaks had a λ_{max} around 510nm which suggested that they were Pg-glycosides, except one (peak 'C') which had a λ_{max} at 524nm and was thought to be a Pn-glycoside. All except one of these anthocyanins were relatively minor components, with the major peak (anth-3) eluting at 27.2 (Table 3.6). The retention time of anth-3 did not correspond to any of the available standards, so anth-3 was not identified by this method.

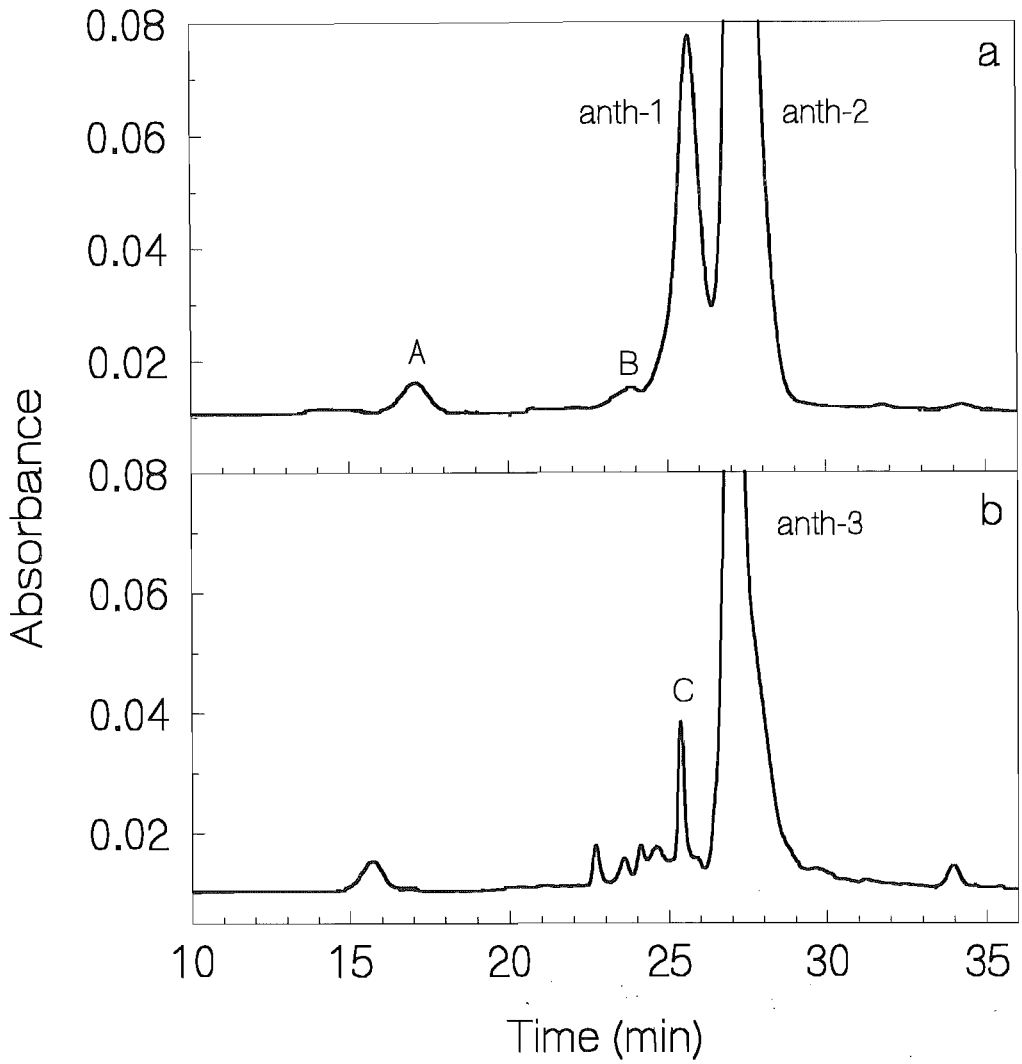


Figure 3.3 HPLC chromatograms (absorbance 530nm) of a) Urenika and b) Red Flesh tuber anthocyanins.

The HPLC chromatogram from the medium purple Arran Victory tubers (not shown) was similar to that of Urenika (Figure 3.3a) except that only one major peak eluting at 25.7min (anth-4) was present (Table 3.6). Chromatograms of extracts from purple-skinned tubers typically followed a pattern similar to that shown by Urenika (two major peaks eluting at 25.4min and 27.3min), or to that shown by Arran Victory (one major peak eluting at 25.7min).

Table 3.6 Chromatographic properties of tuber anthocyanins purified from preparative HPLC.

Cultivar	Name	Retention time	Rf BAW (x100)	Rf 15% HOAc (x100)
Urenika	anth-1	25.4	47.1	52.2
Urenika	anth-2	27.3	52.8	58.5
Red Flesh	anth-3	27.2	59.5	63.0
Arran Victory	anth-4	25.7	46.3	51.6

It was uncertain as to whether or not anth-1 and anth-4 were the same anthocyanin because, although both had similar retention times (Table 3.6) and λ_{max} values of 538nm (Table 3.7), this could have arisen from more than one aglycone or sugar pattern. Both anth-2 and anth-3 eluted at the same retention time (Table 3.6) but, anth-2 from Urenika had a λ_{max} of 538nm, whilst anth-3 from Red Flesh had a λ_{max} of 512nm (Table 3.7), so clearly these were different anthocyanins.

Table 3.7 Spectral analysis of purified anthocyanins.

	anth-1	anth-2	anth-3	anth-4
visible λ_{max} (nm)	538	538	512	538
aglycone ID	Mv, Pt or Dp	Mv, Pt, or Dp	Pg	Mv, Pt or Dp
acyl λ_{max} (nm)	308	306	312	310
acyl group	*p*-coumaric acid	*p*-coumaric acid	*p*-coumaric acid	*p*-coumaric acid
$A_{440}/A_{\lambda max}$ (%)	11.2	8.9	20.0	10.8
5-hydroxyl	substituted	substituted	substituted	substituted

It was decided to focus on these four anthocyanins (anth-1 to anth-4) to purify and identify them. This was because a survey of tuber anthocyanins from other cultivars revealed these four to be the only major anthocyanins present. Subsequently, these major anthocyanins from Urenika, Red Flesh and Arran Victory were purified by preparative HPLC. Each anthocyanin was checked for purity by TLC in BAW and also by analytical HPLC, and their chromatographic properties are summarised in Table 3.6.

When dried, anth-1 and anth-4 were a blue/purple colour and had only limited solubility in methanol, water, or mixtures of these, although they were soluble in both methanol and/or water when acetic acid (or TFA) was added. These similar properties suggested

that they may be the same anthocyanin. Anth-2 was a dark purple colour and anth-3 a red colour, and both were soluble in methanol and water with, and without, the addition of acid. The spectrum of each anthocyanin was recorded in methanol to determine the λ_{max} value, whether a substituted 5-hydroxyl group was present and the identity of any acyl groups (Table 3.7). As an example of this, the spectrum of anth-3 in 15% acetic acid in methanol is shown in Figure 3.4 and shows a typical anthocyanin spectrum with a UV λ_{max} at 280nm and a visible λ_{max} between 500 and 550nm. The visible λ_{max} of this anthocyanin (anth-3), was at 512nm which indicates that the aglycone of this anthocyanin must be Pg. The peak at 312nm suggested the presence of a *p*-coumaric acid acyl group. The A_{440}/A_{512} ratio was 20.0% (Table 3.7), which (for Pg) suggested that the 5-hydroxyl group was substituted (Ribéreau-Gayon, 1972). Spectra were also measured after the addition of $AlCl_3$, to determine if there were any *o*-dihydroxyl groups, to aid in the determination of the aglycone identity.

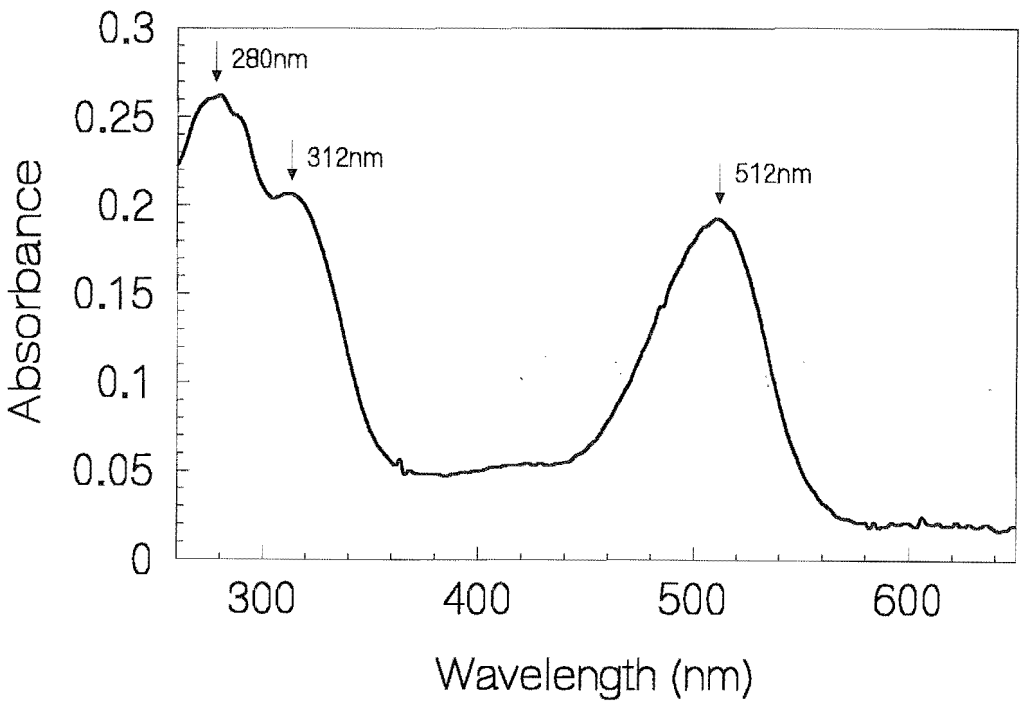


Figure 3.4 Spectrum of anth-3.

The purified anthocyanins were hydrolysed in acid and the aglycones and sugars separated. The aglycones hydrolysed from these anthocyanins were analysed by TLC and compared with aglycone standards run on the same TLC plate (Table 3.8). From these results it was found that both anth-1 and anth-4 contained Pt, anth-2 contained Mv and anth-3 contained Pg.

Table 3.8 Chromatographic and spectral properties of purified anthocyanin aglycones.

Aglycone	λ_{max}	Rf (x100) Forestal(*)	Rf (x100) BAW(*)	Rf (x100) 15% HOAc	Colour visible	Colour UV	Probable identity
anth-1	548	38.4	50.4	-	purple	purple	Petunidin
anth-2	548	53.1	58.7	64.1	purple	purple	Malvidin
anth-3	526	62.2	87.7	72.9	orange/pink	orange/pink	Pelargonidin
anth-4	548	38.1	47.5	-	purple	purple	Petunidin
Pelargonidin	524	62.2 (61)	87.7 (82)	70.8	orange/pink	orange/pink	-
Cyanidin	538	43.4 (45)	71.0 (68)	66.0	pink/purple	pink/purple	-
Delphinidin	546	25.2 (30)	38.0 (42)	58.0	purple	purple	-
Malvidin	548	54.5 (58)	63.4 (56)	63.4	purple	purple	-
Petunidin	548	37.6 (43)	48.3 (52)	48.3	purple	purple	-
Peonidin	542	59.1 (63)	70.0 (71)	69.9	purple	pink	-

* Data in brackets is taken from Strack and Wray (1989)

From their chromatographic and spectral properties (Tables 3.6, 3.7 and 3.8) it was deduced that anth-1 and anth-4 were identical, so all further analysis was carried out only on anth-1.

Time course analysis of the acid hydrolysis was used to give an indication of the glycosylation pattern of the anthocyanins. Figure 3.5 shows a TLC of a typical time course hydrolysis for anth-3, with samples taken at 0, 15, 30, 60, and 120min. At the start of hydrolysis only the dark pink unhydrolysed glycoside (G) was present, with an Rf value of 63.3. After 15min of acid hydrolysis there were two spots visible on the TLC, G and H1. Spot G had faded to a medium pink colour, whilst the additional spot (H1) (Rf = 42.2) was a faint pink colour. After 30min four spots were visible; a medium pink spot G, a medium pink coloured H1, a faint coloured pink spot at Rf 50.0 (H2), and the faint pink Pg aglycone (A) with an Rf value of 84.4. After 60min the same four anthocyanins were present except that spots G and H1 were lighter still, whilst spots H2 and A were darker in colour. After 120min only the Pg aglycone was visible, suggesting that complete hydrolysis of the anthocyanin had occurred. It was expected that the polar unhydrolysed glycoside (G) would have the smallest Rf value, with the Rf value increasing as the polar sugars were removed, leaving the non-polar aglycone (A) with the highest Rf value, because BAW is a fairly non-polar solvent. However, the two hydrolysed glycosides, H1 and H2, had lower Rf values than the unhydrolysed glycoside, G. It was thought that this was because the glycoside (G) contained a non-polar acyl-

group, which was the first substituent group to be lost, exposing the more polar sugars (H1), so that the Rf value decreased. Removal of another sugar (H2) decreased the polarity and increased the Rf. As expected, the aglycone A had the highest Rf value. This time-course of hydrolysis suggests that anth-3 was composed of Pg, an acyl group and two sugars. However, more than one acyl group or more than two sugars may have been present because these compounds may not be hydrolysed from the glycoside individually if they are part of a chain and both attached at a single carbon of the aglycone.

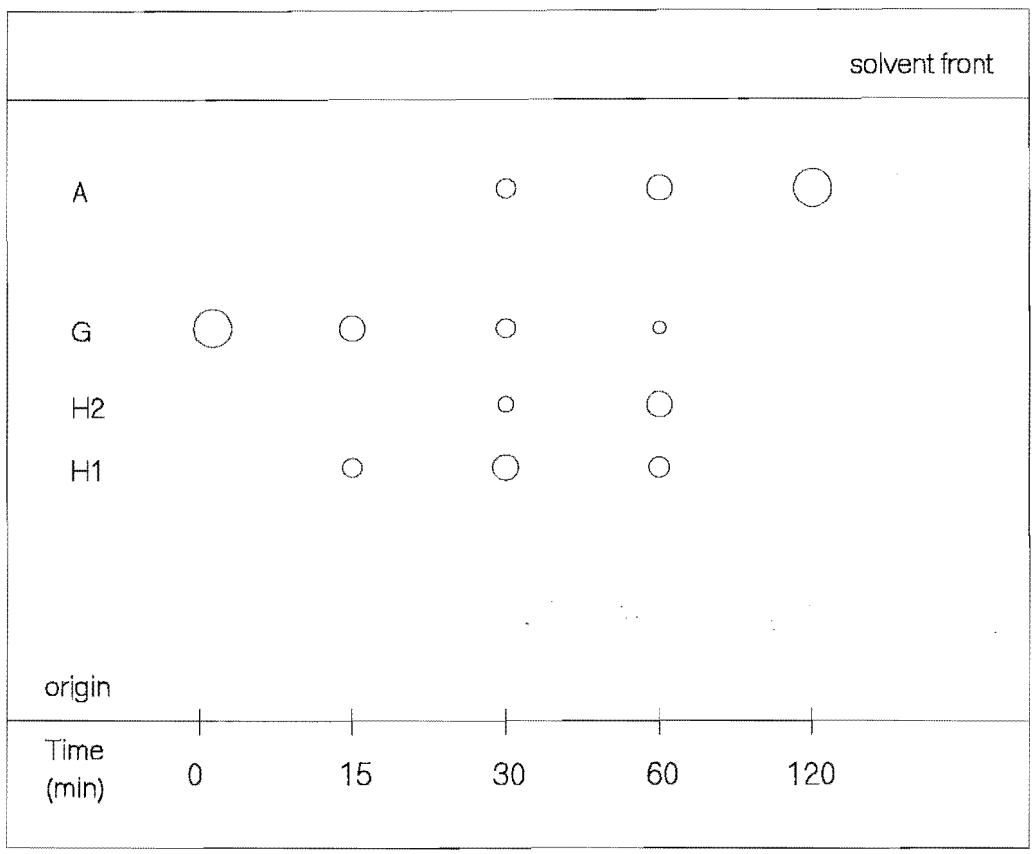


Figure 3.5 TLC of anthocyanin (anth-3) acid hydrolysis time course, developed in BAW. G = unhydrolysed glycoside, H1 = hydrolysed glycoside 1, H2 - hydrolysed glycoside 2, A = aglycone.

A similar acid hydrolysis pattern was observed for anth-1 and anth-2, except that complete hydrolysis took four hours for both of these anthocyanins, and an additional spot was present for anth-1 hydrolyses. Therefore, all these anthocyanins (anth-1, anth-2 and anth-3) contained an acyl group, and anth-1 had at least three sugars attached whilst anth-2 and anth-3 had at least two sugars attached. Time course of acid hydrolysis of anth-4 confirmed that this anthocyanin had the same glycosylation pattern as anth-1.

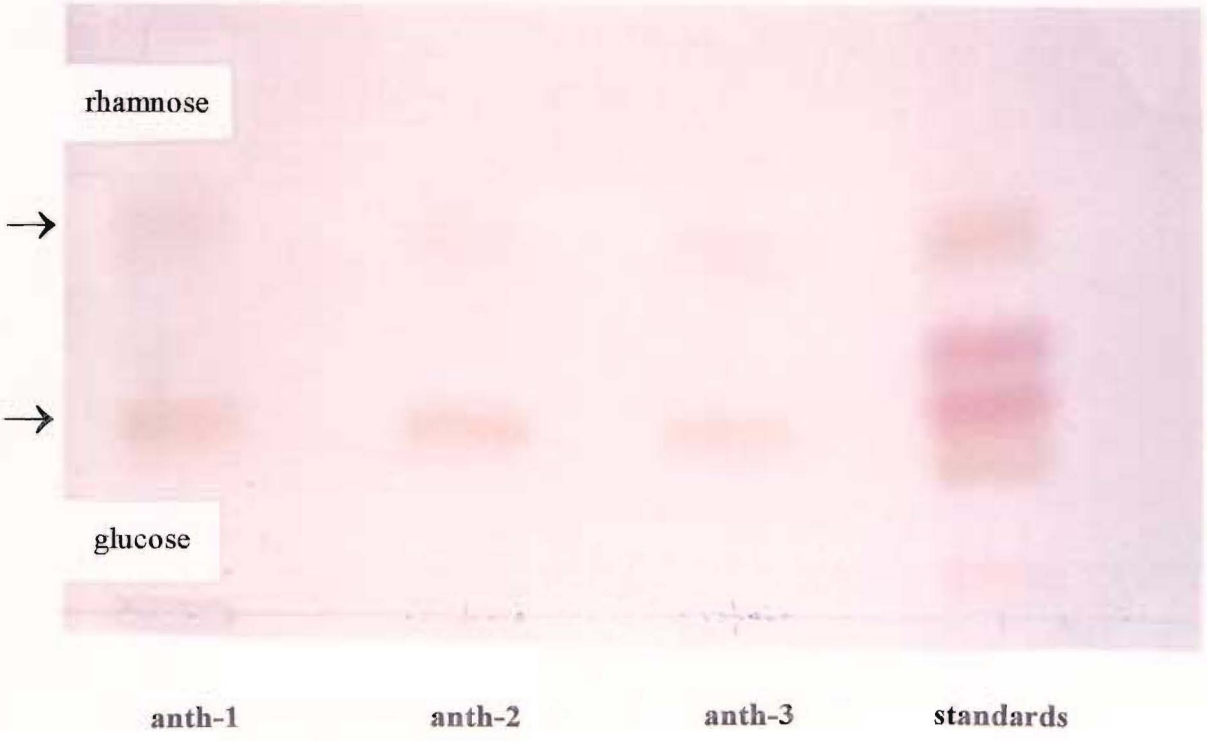


Figure 3.6 TLC of sugars hydrolysed from anth-1, anth-2 and anth-3.

Analysis of the hydrolysed sugars from anth-1, anth-2 and anth-3 was carried out by TLC, developed in BBPW and sprayed with aniline phthalate. The Rf values were calculated and compared with sugar standards run on the same plate (Figure 3.6), which showed that anth-1, anth-2 and anth-3 all contained the sugars glucose and rhamnose.

Anth-1, anth-2 and anth-3 were also investigated by nmr and MS to provide structural evidence to complement the chromatographic and spectral evidence. ^{1}H-nmr was carried out on anth-2 and anth-3. The nmr spectra showed impurities and contaminates from the C_{18}-column, but confirmed that in both cases a phenolic acyl group was present.

FAB-MS was carried out on anth-1, anth-2 and anth-3. Anth-1 proved recalcitrant and was hard to resolve, even after many attempts, so no reliable spectrum was obtained.

The molecular weight (MW) of anth-2 was found to be 947.3. From element composition analysis it was found that the most likely element composition was C 44 H 51 O 23. From the fragmentation spectrum it was concluded that there was:-

1) a peak with a MW of 785.2 which corresponded to anth-2 minus glucose,

2) a peak with a MW of 331.0 which was the MW of Mv, and
3) a peak with a MW of 493.1 which corresponded to Mv plus glucose.

These results indicate that at least one of the glucose units must be attached in such a way as to be able to be cleaved off singly, whilst the other glucose unit must be attached directly to the aglycone. These MS data, combined with information from the above chromatographic and spectral experiments, suggest that the structure of anth-2 was Mv-$(glu)_2$-rha-*p*-coumaric acid. Harborne (1960a; 1967) has indicated that the only structure with these constituents that has been found in potato tubers is Mv-3-(*p*-coumaroyl-rut)-5-glu, therefore it is likely that anth-2 was this compound. This is supported by the fact that the 5-hydroxyl was substituted and this also fitted the fragmentation data. It is suggested that the glucose at the 5-position was cleaved initially leaving the 785.2 MW compound. The *p*-coumaric acid and rhamnose were cleaved subsequently, either separately or together, leaving the glucose attached to the Mv at the 3-position to be cleaved later.

The MW of anth-3 was determined as 887.3. Element composition analysis gave the most likely element composition to be C 42 H 47 O 21. The fragmentation spectrum of anth-3 showed that there was:-

1) a peak with a MW of 725.1 which corresponded to anth-3 minus glucose,
2) a peak with a MW of 270.9 which was the MW of Pg, and
3) a peak with the MW of 433.2 which corresponded to Pg plus glucose.

These MS data combined with information from the above experiments suggest that the structure of anth-3 was Pg-$(glu)_2$-rha-*p*-coumaric acid. From the literature (Harborne, 1960a; 1967) as above, it was likely that anth-3 was Pg-3-(*p*-coumaroyl-rut)-5-glu, because this is the only structure with these constituents that has been found in potato tubers. The ionisation pattern of anth-3 followed that suggested above for anth-2.

The Pn-glycoside found in Red Flesh tubers (peak C in Figure 3.3) was also identified by similar methods (purification from HPLC, spectral analysis, acid hydrolysis and analysis by TLC) as these other anthocyanins (anth-1, anth-2 and anth-3), and was determined to be Pn-3-(*p*-coumaroyl-rut)-5-glu.

In conclusion, the major anthocyanin(s) in the tubers of these cultivars were:-

Urenika (purple/black)	Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1) and Mv-3-(*p*-coumaroyl-rut)-5-glu (anth-2),
Arran Victory (medium purple)	Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-4 = anth-1),
Red Flesh (red)	Pg-3-(*p*-coumaroyl-rut)-5-glu (anth-3) and Pn-3-(*p*-coumaroyl-rut)-5-glu.

3.4.2 Identification of flavonoids and phenolic acids from tubers

The initial investigation of tuber flavonoids by 2-D TLC showed that skin extracts from the different cultivars all contained a similar flavonoid pattern. Flesh extracts contained some of the same compounds but lacked a few of the spots. The initial investigation of Urenika skin extract by 2-D TLC visualised by UV light showed the presence of twelve compounds (Figure 3.7). The spots were tentatively identified by calculating the Rf values and noting the colours of spots under UV light before and after exposure to NH_3, and after spraying with NA-reagent and sodium carbonate, and by comparison with standards (Appendix 2). These results are summarised in Table 3.9.

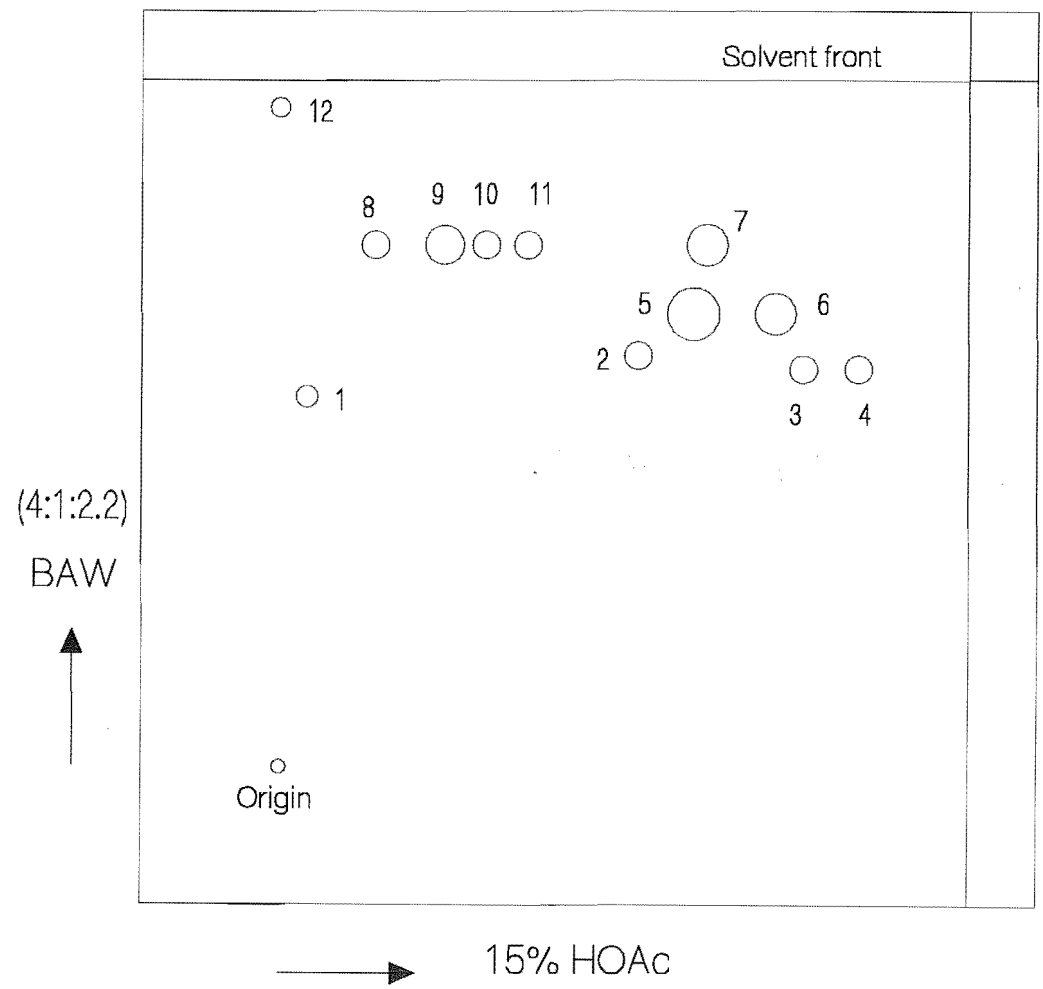


Figure 3.7 Two-dimensional TLC of Urenika skin flavonoids.
Note that the Rf values, colour and identities (where known) of these spots are given in Table 3.9.

Table 3.9 Chromatographic characteristics of flavonoids on 2-D TLC from Urenika skin.

Spot no	Rf (a)	Rf (b)	Colour of spot				Tentative identification
			(1)	(2)	(3)	(4)	
1	53.6	4.2	orange	orange	orange	orange	quercetin
2	60.1	51.6	blue	green	blue	green	phenolic
3	58.3	76.4	blue	blue	blue	blue	phenolic
4	58.7	80.7	blue	blue	blue	blue	phenolic
5	64.8	59.6	blue	yellow	yellow	yellow	kaempferol-glycoside
6	66.0	72.5	blue	green	green	green	chlorogenic acid
7	75.9	62.6	blue	blue	blue	blue	protocatechuic acid
8	77.0	15.0	blue	blue	blue	blue	phenolic
9	76.7	23.4	blue	blue	blue	blue	phenolic
10	75.5	30.4	blue	blue	blue	blue	sinapic acid
11	75.1	36.3	blue	blue	blue	blue	ferulic acid
12	95.1	0.0	orange	orange	yellow	yellow	kaempferol

(a) Rf (x100) BAW

(b) Rf (x100) 15% acetic acid

(1) Colour of spot under UV light

(2) Colour of spot under UV light after exposure to NH_3 fumes

(3) Colour of spot after spraying with NA-reagent, viewed under UV light

(4) Colour of spot after spraying with sodium carbonate, viewed under UV light

The two spots which appeared orange under UV light (spots 1 and 12) were flavonols. Spot 1 remained orange after spraying with NA-reagent, which indicated that it was a 3',4'-dihydroxyflavonol, probably Qu, whereas spot 12 changed to a yellow colour, indicating that it was a 4'-hydroxyl flavonol, probably Km. Spot 5 also gave a yellow colour after spraying with NA-reagent and was probably a Km-glycoside (Table 3.9). Spots 6, 7, 10 and 11 were identified as chlorogenic acid, protocatechuic acid, sinapic acid and ferulic acid respectively. The other spots were likely to be phenolic in nature because of their blue fluorescence in UV light and reactions with the spray reagents; however most of them remain unidentified.

Tuber samples were also analysed by diode-array HPLC. Typical chromatograms from the HPLC analysis of skin (Urenika) and flesh (Arran Victory) are shown in Figures 3.8 and 3.9 respectively. Possible identifications were made by comparing the spectra and retention times of sample peaks with those of authentic standards.

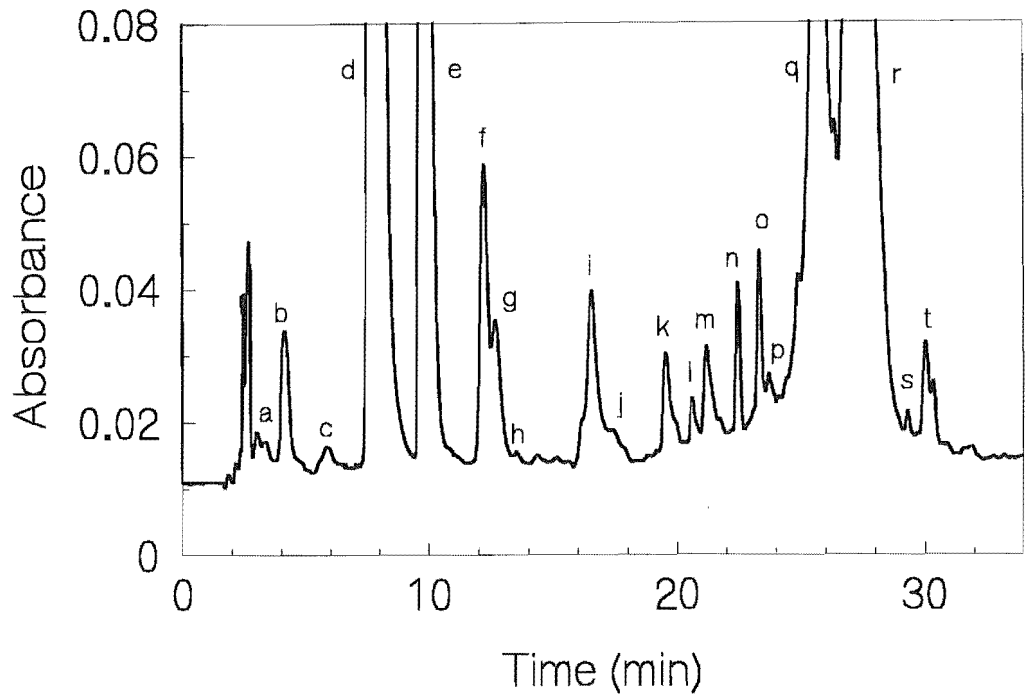


Figure 3.8 HPLC chromatogram of Urenika skin phenolics at 280nm.
Note that the retention times, spectral peaks and identities of these peaks are given in Table 3.10.

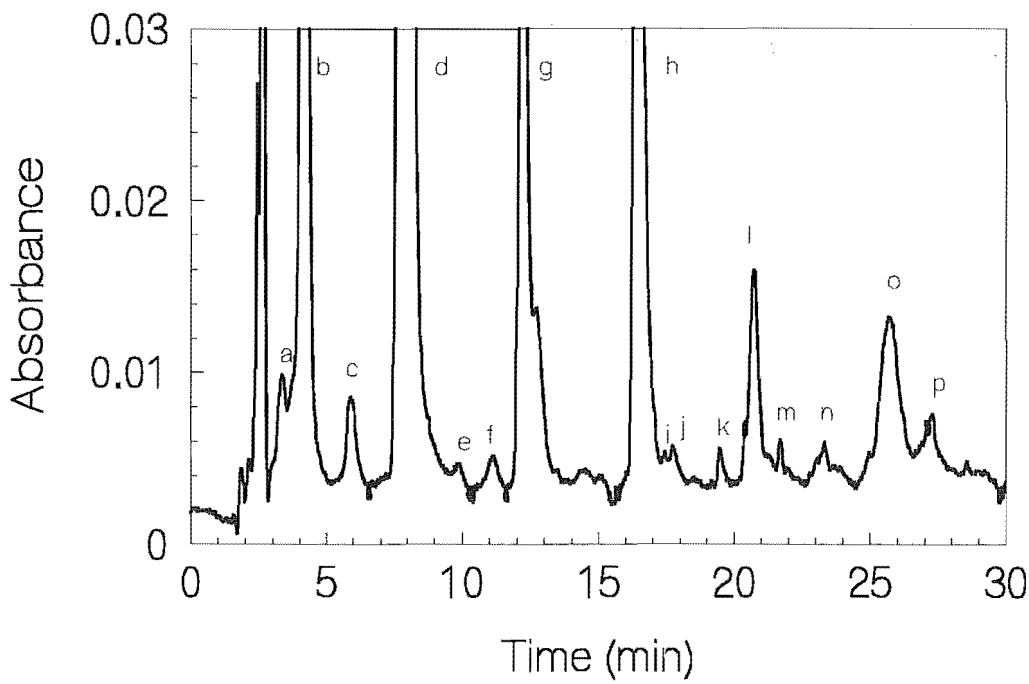


Figure 3.9 HPLC chromatogram of Arran Victory flesh phenolics at 280nm.
Note that the retention times, spectral peaks and identities of these peaks are given in Table 3.11.

For ease of presentation in this thesis these chromatograms (Figures 3.8 and 3.9) show the data at 280nm (rather than at 280, 313, 350 and 530nm), because all phenolics have an absorbance at this wavelength. However, for analysis and calculation of concentrations, the wavelength used depended on the spectrum of the individual compound. Therefore, some phenolic acids, such as cinnamic acid and syringic acid with a λ_{max} between 260nm and 300nm, were measured at 280nm, other phenolic acids, such as chlorogenic acid and *p*-coumaric acid with a λ_{max} between 300nm and 330nm were measured at 313nm. The flavonols with a peak between 340nm and 370nm were measured at 350nm, and the anthocyanins with a peak in the visible range of the spectrum were measured at 530nm. Because these compounds have such varying spectra, the peak area (as shown in the chromatogram at 280nm, Figures 3.8 and 3.9) is not an absolute measurement of concentration because the extinction coefficient varies with the compound and the wavelength. Therefore, some components, especially the flavonols, are under-represented in Figures 3.8 and 3.9.

Table 3.10 Spectral and chromatographic data of Urenika skin phenolics (from Figure 3.8) (sh = shoulder).

Peak	Retention time	Spectral peaks	Identity
a	3.2	270	gallic acid
b	4.0	260, 295	protocatechuic acid
c	5.9	280	catechin
d	7.9	300sh, 325	chlorogenic acid
e	9.8	300sh, 325	caffeic acid
f	12.0	320	unknown phenolic (UP1)
g	12.6	260, 290	vanillic acid
h	13.6	275	syringic acid
i	16.3	300sh, 315	*p*-coumaric acid
j	17.5	255, 350	flavonoid 2b
k	19.4	295sh, 320	ferulic acid
l	20.6	315	unknown phenolic (UP2)
m	21.0	320	sinapic acid
n	22.2	300	salicylic acid
o	23.1	280, 320sh	eriodictyol
p	23.8	300sh, 320	unknown phenolic (UP3)
q	25.5	280, 540	anth-1
r	27.1	280, 540	anth-2
s	29.3	280	cinnamic acid
t	30.0	290, 325sh	naringenin

Table 3.11 Spectral and chromatographic data of Arran Victory flesh phenolics (from Figure 3.9). (sh = shoulder).

Peak	Retention time	Spectral peaks	Identity
a	3.3	270	gallic acid
b	4.1	260, 295	protocatechuic acid
c	5.9	280	catechin
d	7.8	300sh, 325	chlorogenic acid
e	9.9	300sh, 325	caffeic acid
f	11.0	280	epicatechin
g	12.2	320	unknown phenolic (UP1)
h	16.4	300sh, 315	*p*-coumaric acid
i	17.2	255, 350	flavonoid 2a
j	17.8	255, 350	flavonoid 2b
k	19.5	295sh, 320	ferulic acid
l	20.6	315	unknown phenolic (UP2)
m	21.5	320	sinapic acid
n	23.2	280, 320sh	eriodictyol
o	25.5	280, 540	anth-1
p	27.2	280, 540	anth-2

Table 3.10 shows the data for the skin extract (Urenika) chromatogram and Table 3.11 for the flesh (Arran Victory) sample. Tubers (skin and flesh) appeared to contain mostly phenolic acids with only small amounts of flavonoids (other than anthocyanins). The major phenolic acid present in tuber skin (e.g. Urenika Figure 3.8, Table 3.10) was chlorogenic acid (peak d), followed by caffeic acid (peak e). Other phenolic acids present in moderate amounts included protocatechuic acid (peak b), vanillic acid (peak g), *p*-coumaric acid (peak i), ferulic acid (peak k), sinapic acid (peak m), salicylic acid (peak n) and an unidentified phenolic (UP1, peak f). Also present in moderate quantities were the flavanones, eriodictyol (peak o) and naringenin (peak t). Low concentrations of catechin (peak c), syringic acid (peak h), flavonoid 2b (peak j), cinnamic acid (peak s) and the unknown phenolic acids, UP2 (peak l) and UP3 (peak p) were found. Because of the high concentrations of anthocyanins, anth-1 (peak q) and anth-2 (peak r), and their absorbance at 280nm (Figure 3.8), some compounds at low concentrations that co-eluted with these anthocyanins may not have been detected. Therefore, the chromatograms of skin extracts from white-skinned tubers (with the absence of these anthocyanins) were studied and showed that My and an unknown flavonoid (UF2) were present in the extracts from white-skinned tubers (not shown). It is likely that the extracts from coloured tubers also contained My and UF2, but that the peaks representing these

compounds were obscured by the anthocyanin peaks. In extracts from white-skinned tubers these two flavonoids (My and UF2) accounted for an average of 7.5% of the total flavonoids (excluding anthocyanins) (Appendix 3, Table A3.2), and are expected to have existed in similar concentrations in the extracts from tubers with coloured skins. None of the flesh extracts from any of the white-fleshed tubers analysed contained My or UF2.

In general, tuber flesh (e.g. Arran Victory Figure 3.9, Table 3.11) contained fewer phenolics than the skin and again, the major phenolic acid present was chlorogenic acid (peak d). However, in contrast to the skin samples, only small amounts of caffeic acid were present (peak e). Phenolic acids present in the tuber flesh in moderate amounts (Figure 3.9, Table 3.11) were protocatechuic acid (peak b), *p*-coumaric acid (peak h), ferulic acid (peak k), and two unknown phenolics, UP1 (peak g) and UP2 (peak l). Phenolic acids present in tuber flesh at low concentrations were gallic acid (peak a) and sinapic acid (peak m), and flavonoids present at low concentrations included catechin (peak c), epicatechin (peak f), flavonoid 2a (peak i), flavonoid 2b (peak j) and eriodictyol (peak n). Most extracts of tuber flesh contained no anthocyanins, but Arran Victory contained anth-1 (peak o) and anth-2 (peak p).

As well as the unknown phenolic acids and flavonoids noted (Tables 3.10 and 3.11), there were also a few very minor peaks which remained unidentified (Figures 3.8 and 3.9), but these compounds represented less than two percent of the total phenolic content and therefore were not included in further analyses. From their spectra most of these were thought to be phenolics, although in some cases there was so little compound present that a clear spectrum was unobtainable.

3.4.3 Identification of anthocyanins from flowers

Initial investigation of flower anthocyanins was carried out by 2-D TLC. Up to three anthocyanins were visible in the flower extracts from those cultivars with dark coloured flowers (Figure 3.10). Extracts from lighter coloured flowers showed mixtures of these same three anthocyanins, although the pigments on TLC were absent or fewer in number from the lighter coloured flowers. Therefore, the extracts from the darker coloured flowers were used to identify the anthocyanins present in flowers.

The chromatographic properties of these flower anthocyanins are shown in Table 3.12. Acid hydrolysis, as carried out for the tuber anthocyanins (Section 3.4.1), indicated that Pg, Pt and Mv were the major aglycones, and it was thought, from this TLC analysis, that the three main anthocyanins in flowers were anth-1 (Pt-3-(*p*-coumaroyl-rut)-5-glu), anth-2 (Mv-3-(*p*-coumaroyl-rut)-5-glu) and a Pg-glycoside.

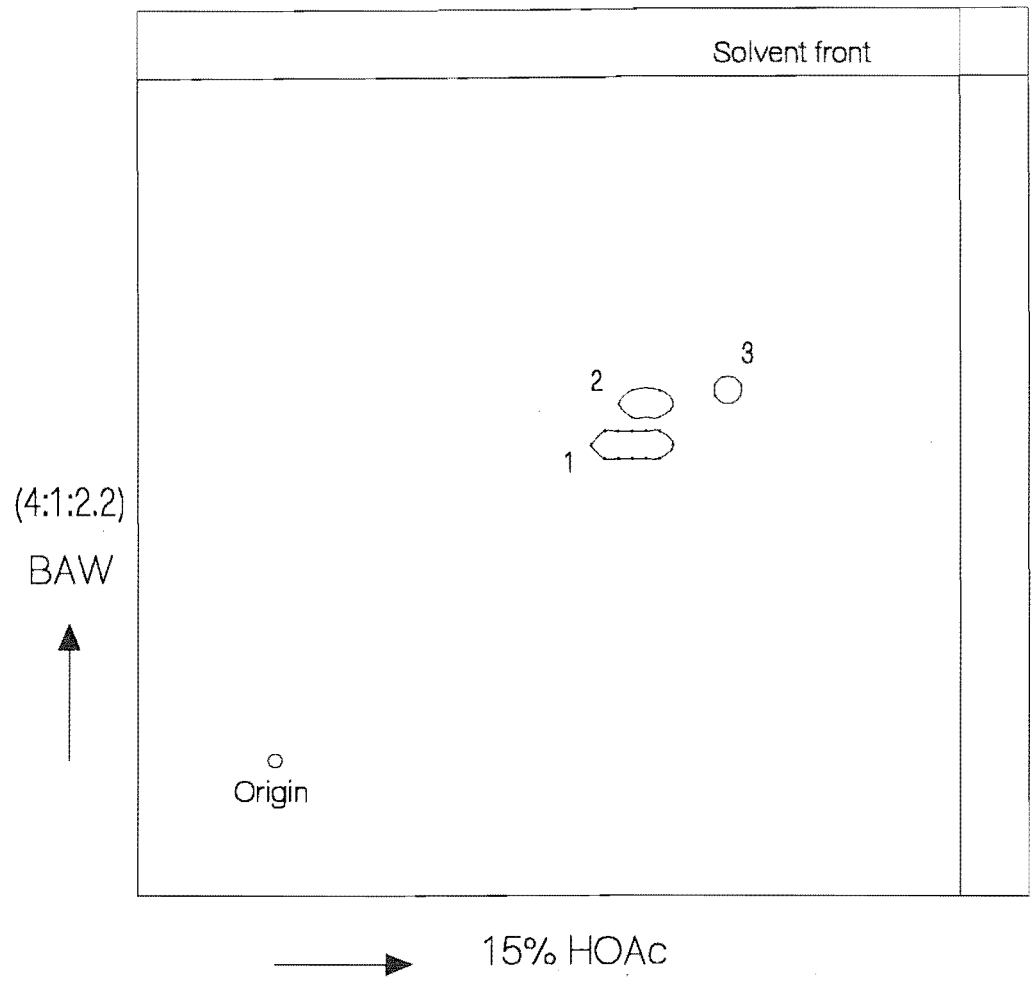


Figure 3.10 Two-dimensional TLC of anthocyanins from a typical dark flower extract. Note that the chromatographic properties of these spots are given in Table 3.12.

Table 3.12 Chromatographic properties of flower anthocyanins from a typical dark coloured flower extract (from Figure 3.10).

Spot number	Rf (x100) BAW	Rf (x100) 15% HOAc	Colour of spot (visible)	Colour of spot (UV)	Probable identity
1	46.4	51.7	blue	purple	anth-1
2	51.7	53.1	red/purple	purple	anth-2
3	53.6	63.6	pink/purple	pink	Pg-gly

A HPLC chromatogram of a strongly coloured flower is presented in Figure 3.11 and showed three major anthocyanins (peaks c, d and e), and other minor anthocyanins (peaks a, b and f) which were present only in some darker coloured flowers.

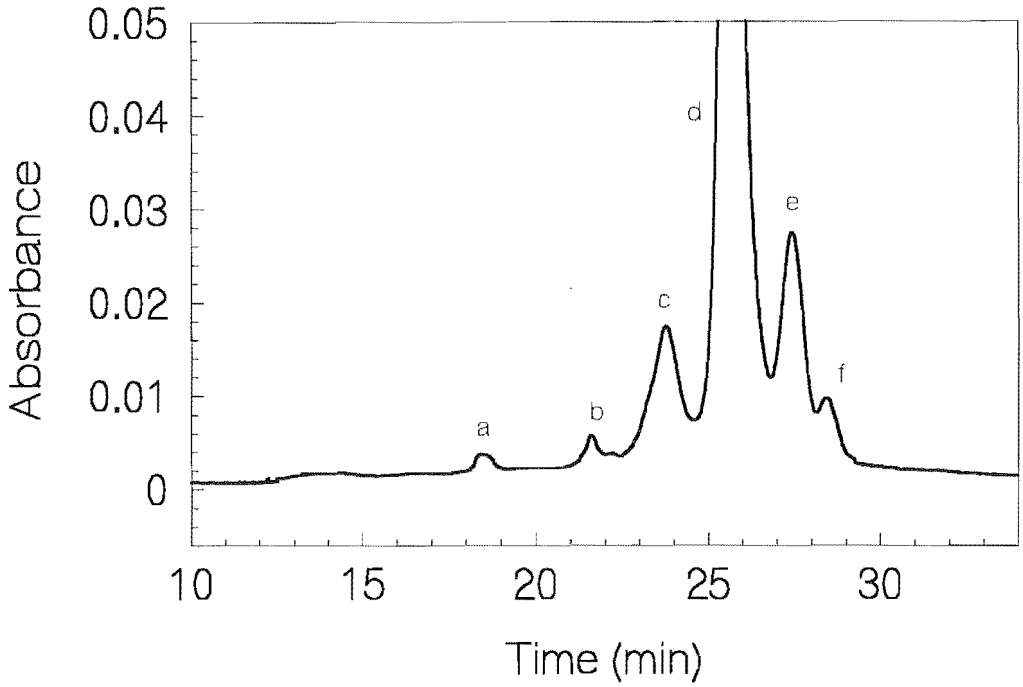


Figure 3.11 HPLC of anthocyanins from a typical dark coloured flower extract shown at 530nm.

The major anthocyanins present in flowers were tentatively identified as Pg-3-rut (peak c), anth-1 (Pt-3-(*p*-coumaroyl-rut)-5-glu, peak d) and anth-2 (Mv-3-(*p*-coumaroyl-rut)-5-glu, peak e), and the minor peaks were tentatively identified as Dp-3-rut (peak a), Cy-3-rut (peak b) and a Pg-glycoside (peak f) (as indicated by the similarity of retention times and spectra to standards) (Figure 3.11 and Table 3.13). The identities of the major anthocyanins determined by HPLC correlated with those determined by TLC methods.

Table 3.13 Tentative identities of flower anthocyanins from a typical dark coloured flower extract (from Figure 3.11).

Peak	Retention time	Tentative identification
a	18.7	delphinidin-3-rutinoside
b	21.3	cyanidin-3-rutinoside
c	24.5	pelargonidin-3-rutinoside
d	25.7	anth-1
e	27.3	anth-2
f	28.0	pelargonidin-glycoside

3.4.4 Identification of flavonoids and phenolic acids from flowers

The initial investigation of flower flavonoids by 2-D TLC and visualisation by UV light showed that fourteen compounds were present (Figure 3.12), and spraying with NA-reagent enabled three more compounds to be detected. The spots were tentatively identified by calculating the Rf values and noting the colours of spots under UV before and after exposure to NH_3, and after spraying with NA-reagent and sodium carbonate, and by comparison with standards (Appendix 2). These results are summarised in Table 3.14. Most of the spots (spot numbers 1 to 9 and 13 to 17) appeared to be flavonoids, spot 10 was chlorogenic acid and the other two (spots 11 and 12) were unidentified phenolics.

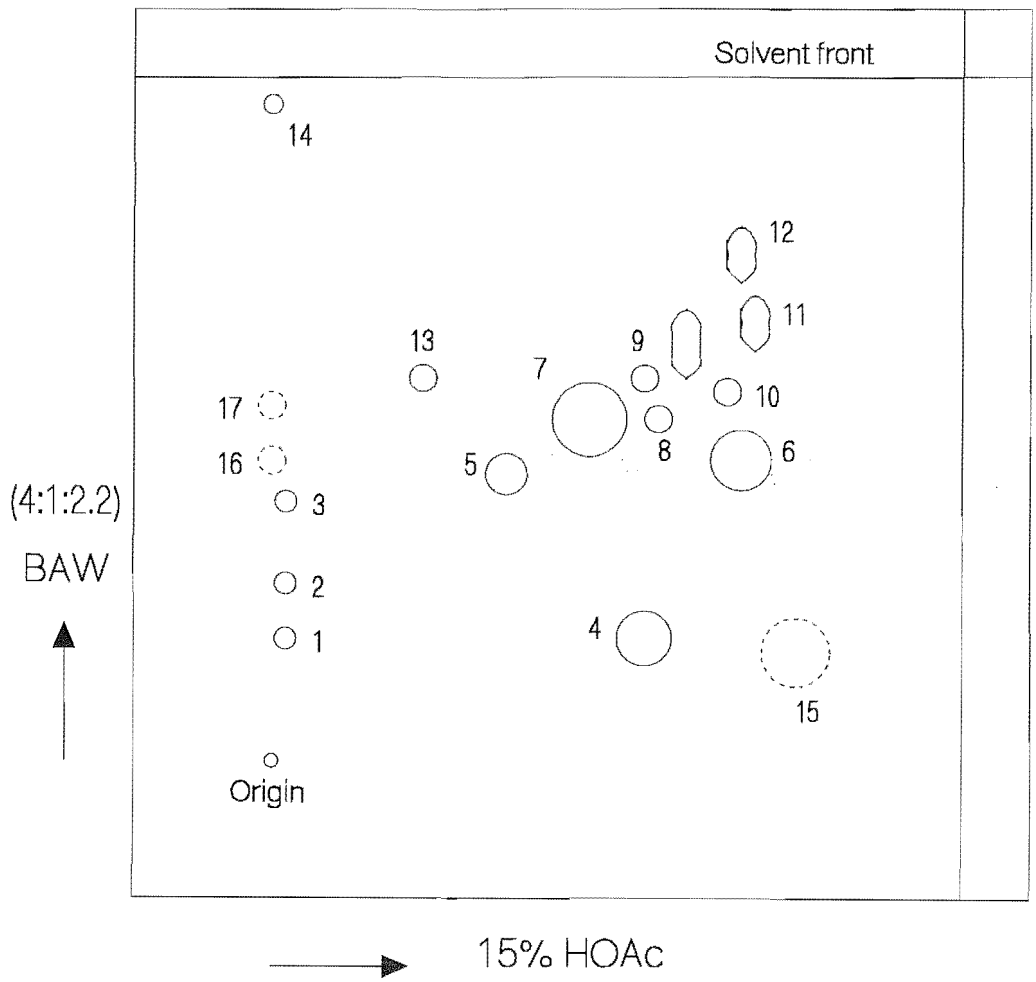


Figure 3.12 Two-dimensional TLC of flower flavonoids. The dotted areas represent spots which were not visible under UV light, but were visible after spraying with NA-reagent. Note that the Rf values, colour and identities (where known) of these spots are given in Table 3.14.

Table 3.14 Chromatographic properties of flower flavonoids (from Figure 3.12).

Spot no	Rf (a)	Rf (b)	Colour of spot (1)	(2)	(3)	(4)	Tentative identification
1	17.8	2.1	orange	orange	orange	-	flavonoid
2	26.5	2.4	orange	orange	orange	-	flavonoid
3	38.7	2.4	orange	orange	orange	-	myricetin
4	18.5	53.3	purple	-	orange	-	flavonoid
5	41.5	33.7	purple	orange	orange	orange	quercetin-glycoside
6	43.5	68.1	purple	orange	orange	orange	quercetin-glycoside
7	50.5	46.7	purple	orange	orange	yellow	kaempferol-3-rutinoside
8	50.5	55.1	purple	orange	orange	orange	rutin
9	55.6	53.3	blue	yellow	yellow	yellow	kaempferol-glycoside
10	61.3	59.6	blue	green	green	green	chlorogenic acid
11	61.2	69.5	blue	blue	yellow	blue	phenolic
12	71.2	68.1	blue	green	yellow	blue	phenolic
13	56.6	21.4	purple	orange	orange	-	quercetin-3-glucoside
14	96.5	0	yellow	yellow	orange	-	kaempferol
15	15.1	76.1	-	-	orange	-	flavonoid
16	43.0	0	-	-	orange	-	flavonoid
17	52.1	0	-	-	orange	-	quercetin

(a) Rf (x100) BAW

(b) Rf (x100) 15% acetic acid

(1) Colour of spot under UV light

(2) Colour of spot under UV light after exposure to NH_3 fumes

(3) Colour of spot after spraying with NA-reagent, viewed under UV light

(4) Colour of spot after spraying with sodium carbonate, viewed under UV light

Analytical HPLC analysis was in general agreement with the 2-D TLC results. A typical HPLC trace of flower flavonoids is shown in Figure 3.13, and the retention times, spectra and peak identities are shown in Table 3.15. Peaks were identified by comparison of retention times and spectra of known standards. The phenolic acids tended to elute off the column first, most of them with retention times between 3 and 15min, whilst the flavonols eluted later with retention times mainly between 21 and 25min. The flower extracts had low concentrations of phenolic acids, except chlorogenic acid (peak e) which was present in large amounts, plus protocatechuic acid (peak b) and the unknown phenolic, UP1 (peak h), which were present in moderate amounts (Figure 3.13). In contrast to the tuber extracts, flower extracts had much higher concentrations of flavonoids, especially flavonoid 2a (peak l), flavonoid 2b (peak m) and rutin (peak q).

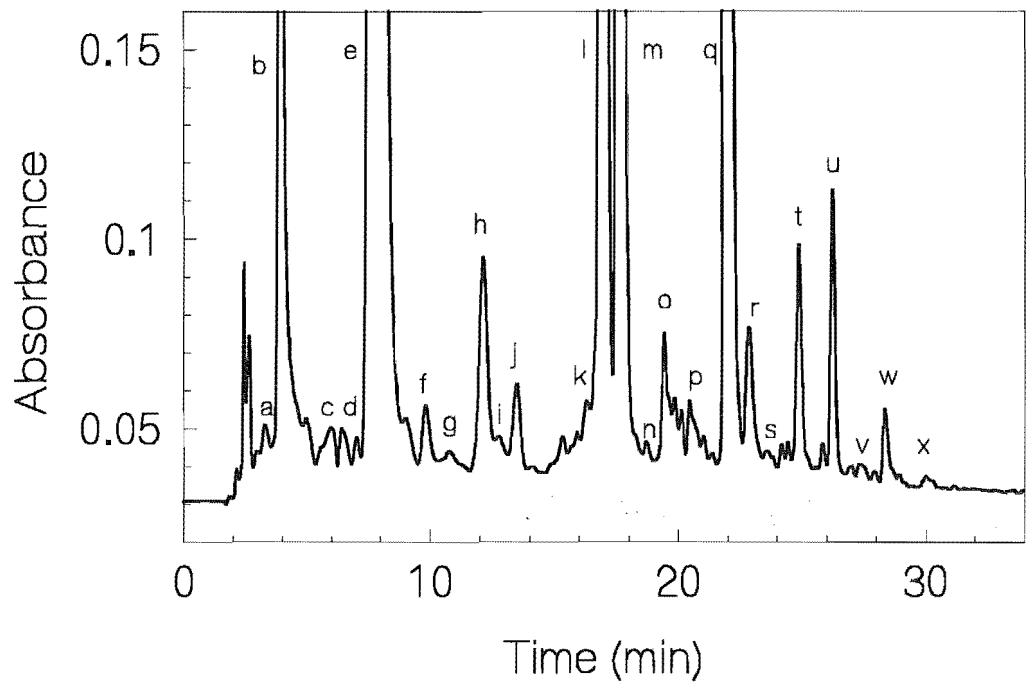


Figure 3.13 Typical HPLC chromatogram of flower flavonoids (from a light coloured flower).

Five peaks from the flower extracts were identified further (Figure 3.13, peaks e, l, m, q and t) because these were major peaks and it was important to either confirm the identity or, in the case of peaks l and m (flavonoids 2a and 2b) to attempt to determine the identity. Flower extracts from a number of cultivars were combined and then the major peaks, detected at 350nm, were separated by preparative RP-18 HPLC. Initially, four major peaks were collected (fractions 1-4), with the second fraction being re-chromatographed into two peaks (fractions 2a and 2b). The purity of these compounds was checked by TLC in BAW. Fractions 1, 2a, and 2b appeared to be pure, although fraction 3 also contained an anthocyanin, and fraction 4 contained a small quantity of fraction 3. Chromatographic properties of these compounds are shown in Table 3.16.

Table 3.15 HPLC data of flower phenolics (from Figure 3.13) (sh = shoulder).

Peak	Retention time	Spectra	Tentative identity
a	3.3	255	gallic acid
b	4.1	260	protocatechuic acid
c	5.9	280	catechin
d	6.3	255	*p*-hydroxybenzoic acid
e	7.8	300sh, 320	chlorogenic acid
f	9.9	300sh, 325	caffeic acid
g	10.9	280	epicatechin
h	12.2	300sh, 320	unknown phenolic (UP1)
i	12.7	260, 290	vanillic acid
j	13.6	280	syringic acid
k	16.6	300sh, 310	*p*-coumaric acid
l	17.2	255, 350	flavonoid 2a
m	17.9	255, 350	flavonoid 2b
n	18.8	265, 350	unknown flavonoid (UF1)
o	19.5	300sh, 325	ferulic acid
p	20.1	260, 350	quercetin-3-galactoside
q	21.9	255, 350	rutin
r	22.9	260, 350	quercetin-3-glucoside
s	24.2	300sh, 325	unknown phenolic (UP3)
t	25.0	270, 350	kaempferol-3-rutinoside
u	25.9	265, 365	myricetin
v	27.3	265, 350	unknown flavonoid (UF2)
w	28.2	280	cinnamic acid
x	30.1	290, 325	naringenin

The retention time and chromatographic properties of fraction 1 (corresponding to peak e in Figure 3.13 and Table 3.15) confirmed that this fraction was chlorogenic acid. The chromatographic properties of the other fractions indicated that they were flavonols however, the retention times of fractions 2a (peak l) and 2b (peak m) were much lower than those of any of the available flavonol standards. These chromatographic properties (Table 3.16) suggested that fractions 2a and 2b were probably Qu-glycosides and that fraction 3 (peak q) and fraction 4 (peak t) were rutin and Km-3-rut, respectively (by comparison with published data (Mabry *et al.*, 1970) and standards (Appendix 2)).

Table 3.16 Chromatographic characteristics of purified flower flavonoids.

Fraction (*)	Retention time	Rf (a)	Rf (b)	Colour of spot (1)	(2)	(3)	(4)	Tentative identity
1 (e)	7.8	64.3	63.0	blue	blue/ green	yellow	green	chlorogenic acid
2a (l)	17.2	44.4	69.0	purple	orange	orange	yellow	quercetin-glycoside
2b (m)	17.9	50.0	53.6	purple	orange	orange	yellow	quercetin-glycoside
3 (q)	21.9	52.0	39.2	purple	orange	orange	yellow	rutin
4 (t)	25.0	58.7	46.4	purple	yellow	orange	yellow	kaempferol-3-rutinoside
rutin standard	21.70	52.0	40.0	purple	orange	orange	yellow	-

(*) corresponding peak from Figure 3.13 and Table 3.15

(a) Rf (x100) BAW

(b) Rf (x100) 15% acetic acid

(1) Colour of spot under UV light

(2) Colour of spot under UV light after exposure to NH_3 fumes

(3) Colour of spot after spraying with NA-reagent, viewed under UV light

(4) Colour of spot after spraying with sodium carbonate, viewed under UV light

These purified flavonoids (fractions 2a, 2b, 3 and 4) were hydrolysed in acid and the aglycones and sugars separated. Fraction 1 was not hydrolysed because it was identified as chlorogenic acid (and was not glycosylated). The hydrolysis was carried out to identify the structural components, especially the aglycone, because further work on the glycosylated flavonoids, such as recording the spectra with the use of shift reagents to determine the position of hydroxyl groups and substitution patterns was easier if the aglycone identity was known.

To determine the identity of the aglycones the spectra (240-500nm) of the hydrolysed fractions of 2a, 2b and 3, and standard aglycones were recorded (Table 3.17). The spectrum of fraction 4 was not measured due to the presence of fraction 3 as an impurity. The comparison of spectra from the fractions with that of standards showed that fractions 2a, 2b and 3 were likely to contain Qu as the aglycone, however the spectrum of My was also very similar (Table 3.17), and fractions may have contained My instead.

Table 3.17 Spectral properties of purified flavonol aglycones after acid hydrolysis (sh = shoulder).

Aglycone	UV spectra λ_{max} (nm)				Tentative identity
2a	256	272sh	300sh	370	quercetin (or myricetin)
2b	256	270sh	302sh	370	quercetin (or myricetin)
3	252	270sh	300sh	370	quercetin (or myricetin)
kaempferol	266	-	322sh	366	
quercetin	256	268sh	304sh	370	
myricetin	254	268sh	302sh	374	

Conformation of the identity of the aglycones was made by determining the Rf values from the TLC analysis of the hydrolysed aglycones and standards. This showed that fractions 2a, 2b and 3 all contained Qu, not My, whilst fraction 4 contained Km (Table 3.18).

Table 3.18 Chromatographic properties of purified flavonol aglycones after acid hydrolysis.

Aglycone	Rf BAW (x100)	Rf Forestal (x100)	Colour of spot (UV)	Identity of aglycone
2a	59.1	28.2	orange	quercetin
2b	58.5	28.2	orange	quercetin
3	55.6	26.0	orange	quercetin
4	77.7	42.9	orange	kaempferol
kaempferol	83.2	45.4	orange	-
quercetin	62.9	27.6	orange	-
myricetin	37.8	14.3	orange	-

To determine the identity of the sugars from the acid hydrolysis, TLC of the sugar fractions in BBPW was carried out and the plates sprayed with aniline phthalate. The Rf values were calculated and compared with sugar standards run on the same plate. This TLC chromatogram (not shown) was similar to that shown in Figure 3.6 and it was determined that fractions 2a, 2b, 3 and 4 all contained the sugars glucose and rhamnose.

To finally confirm the structure of the aglycones and determine the substitution patterns of the glycosylated flavonoids the spectra (240-500nm) of the unhydrolysed fractions 1, 2a, 2b and 3 were recorded, and the λ_{max} and bathochromic shifts with the shift reagents, NaOH, $AlCl_3$, HCl, NaOAc and H_3BO_3, were noted (Table 3.19). The spectrum of fraction 1 was measured only in methanol, since it was a phenolic acid (chlorogenic acid) and the shift reagents apply only to the flavonoids. The spectrum of fraction 4 was not measured due to the presence of fraction 3 as an impurity.

Table 3.19 Spectral properties and shifts of purified flavonoids from flowers.

Fraction	Shift reagent	UV spectra λ_{max} (nm)			
		Band II		Band I	
1	MeOH	-	-	302sh	326
2a	MeOH	256	266sh	308sh	354
	NaOH	d			
	NaOAc	262	-	306sh	360
	NaOAc + H_3BO_3	262	296sh	-	380
	$AlCl_3$	256	-	304sh	356
	$AlCl_3$ + HCl	258	-	300sh	356
2b	MeOH	256	266sh	306sh	356
	NaOH	d			
	NaOAc	266	-	-	392
	NaOAc + H_3BO_3	266	-	308sh	378
	$AlCl_3$	256	-	304sh	430
	$AlCl_3$ + HCl	258	300sh	360sh	398
3	MeOH	258	266sh	302sh	358
	NaOH	d			
	NaOAc	268	310sh	-	406
	NaOAc + H_3BO_3	262	292sh	-	382
	$AlCl_3$	274	300sh	-	430
	$AlCl_3$ + HCl	268	294	360sh	400

sh = shoulder of peak

d = spectrum decomposed immediately

- = peak absent

The spectrum of fraction 1 was identical to that of chlorogenic acid (Appendix 2), confirming the identity of this peak. The addition of NaOH to fractions 2a, 2b and 3 caused the spectrum to decompose immediately suggesting the presence of alkali sensitive groups. However, according to data by Mabry *et al.* (1970), when sodium methoxide was added to rutin in methanol, the spectrum did not decompose, which conflicted with the evidence obtained so far that fraction 3 was rutin. Therefore, NaOH was added to the rutin standard which also decomposed, so the NaOH data was ignored for all fractions. It is thought that this difference may have been caused by using NaOH instead of sodium methoxide as the strong base. It is possible also that the sugars were removed leaving Qu (which contains the alkali-sensitive 3,3',4'-hydroxyl grouping).

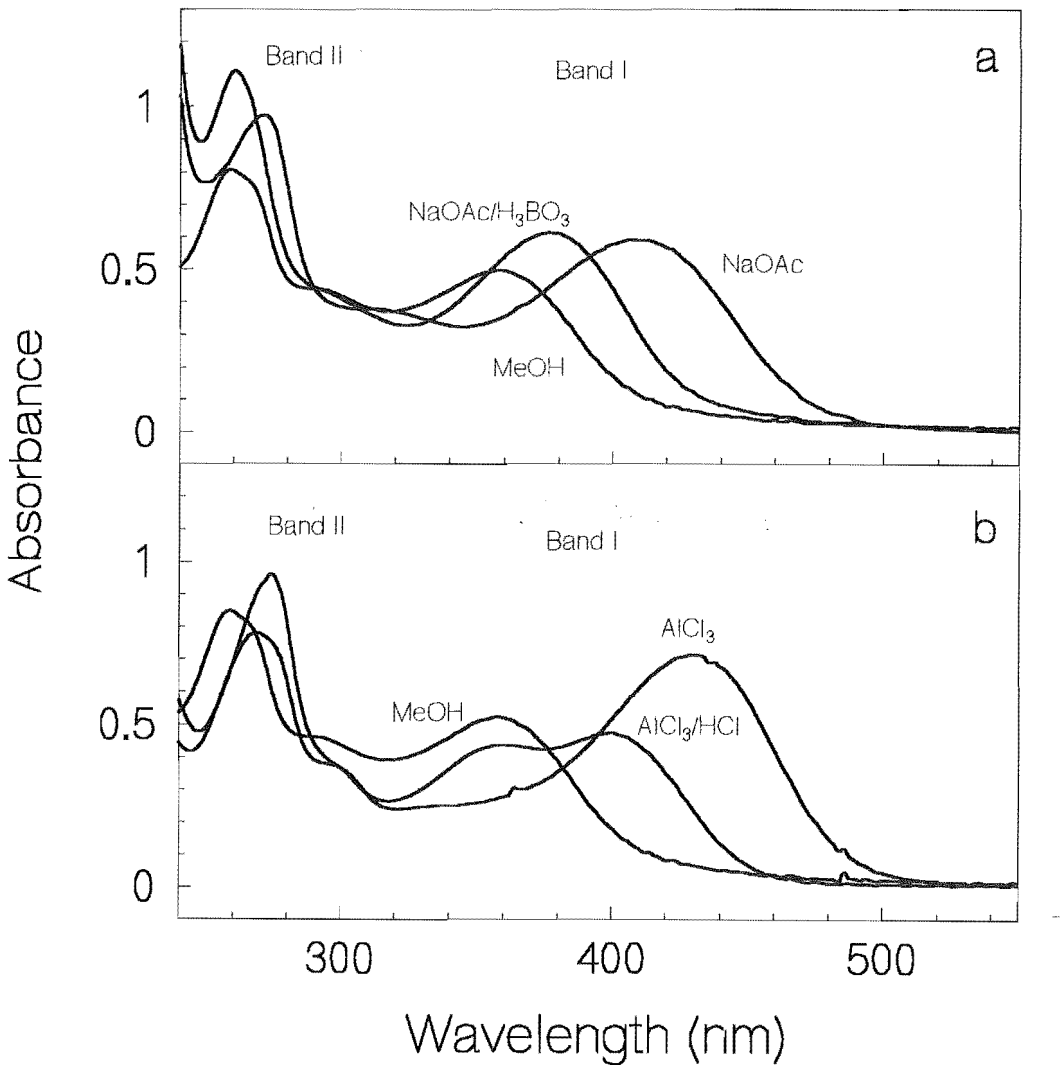


Figure 3.14 a) Spectra of fraction 3 in methanol (MeOH), and with shift reagents NaOAc and $NaOAc/H_3BO_3$. b) Spectra of fraction 3 in methanol (MeOH) and with shift reagents $AlCl_3$ and $AlCl_3/HCl$.

The spectra and reactions with shift reagents of the three fractions (2a, 2b and 3) were all similar so, as an example, the spectra of fraction 3 in methanol and with the shift reagents are shown in Figure 3.14. The methanolic spectra of fractions 2a, 2b and 3 showed them to be flavonols. For example, from the spectrum of fraction 3 in methanol (Figure 3.14) the pronounced Band II indicated oxygenation of the A-ring and the presence of a strong Band I showed that the B-ring was also oxygenated (Markham, 1982).

The hypsochromic shift of Band I from 370nm in the aglycone spectrum (Table 3.17) to 358nm in the unhydrolysed fraction (Table 3.19) indicated that a 3-, 5- or 4'-hydroxyl group was methylated or glycosylated in the unhydrolysed fraction. Since the aglycone has been shown to be Qu, it is known that these hydroxyls were not methylated, and the shift of 12nm suggested that the 3- and/or 5-hydroxyl group was glycosylated.

The shift of Band II from 258nm to 268nm (Figure 3.14a, Table 3.19) upon the addition of NaOAc to the methanolic fraction suggested that fraction 3 contained a free 7-hydroxyl group, and the shift in Band I from 358nm to 406nm confirmed this. The spectrum did not decompose with this weaker alkali, suggesting that fraction 3 did not contain any alkali sensitive groups (such as 5,6,7-; 5,7,8- or 3,3',4'-hydroxyl groups). Thus, the reaction with NaOAc supported the previous finding that the aglycone was Qu.

The bathochromic shift of Band I of 24nm from the methanolic spectrum (358nm) in the presence of $NaOAc/H_3BO_3$ (382nm) (Figure 3.14a, Table 3.19) showed that fraction 3 contained B-ring *o*-dihydroxyl groups. This was confirmed by the hypsochromic shift of 30nm of Band I of the $AlCl_3$ spectrum (430nm) on the addition of HCl (400nm) (Figure 3.12b, Table 3.19). These results supported the identification of the aglycone as Qu. The shift of Band I (and Band II) of 42nm (and 10nm) between the methanolic and the $AlCl_3/HCl$ spectrum indicated that fraction 3 contained a free 3- and/or 5-hydroxyl group which fitted with the rutin structure with sugars attached to the 3-hydroxyl, leaving the 5-hydroxyl free. Finally, the comparison of all the spectra of fraction 3 (except NaOH) with that published for rutin in Mabry *et al.* (1970), together with the previous finding that it contained glucose and rhamnose as sugars, confirmed that fraction 3 was rutin.

In summary, fractions 2a and 2b had a free 7-hydroxyl group, B-ring *o*-dihydroxyl groups and a free 3- and/or 5-hydroxyl group, all of which indicated that these compounds had Qu aglycones. As well, the methanolic spectrum showed that the 3- and/or the 5-hydroxyl group was glycosylated. Neither of the spectral data of these fractions matched that of any of those for Qu-glycosides published in Mabry *et al.* (1970). The presence of acyl groups was not able to be tested for, so fractions 2a and 2b may have contained an acyl group.

From all the evidence cited above, it is proposed that the structure of the purified flower fractions was:- fraction 1 chlorogenic acid
fraction 2a Qu-glu-rha
fraction 2b Qu-glu-rha
fraction 3 rutin
fraction 4 Km-3-rut

Fractions 2a and 2b could not be fully identified and, although the composition was determined, the attachment positions of the sugars and presence of an acyl group was not elucidated. However, both fractions 2a and 2b were found to contain Qu as an aglycone with at least one glucose and one rhamnose attached in the 3- and/or 5-hydroxyl positions.

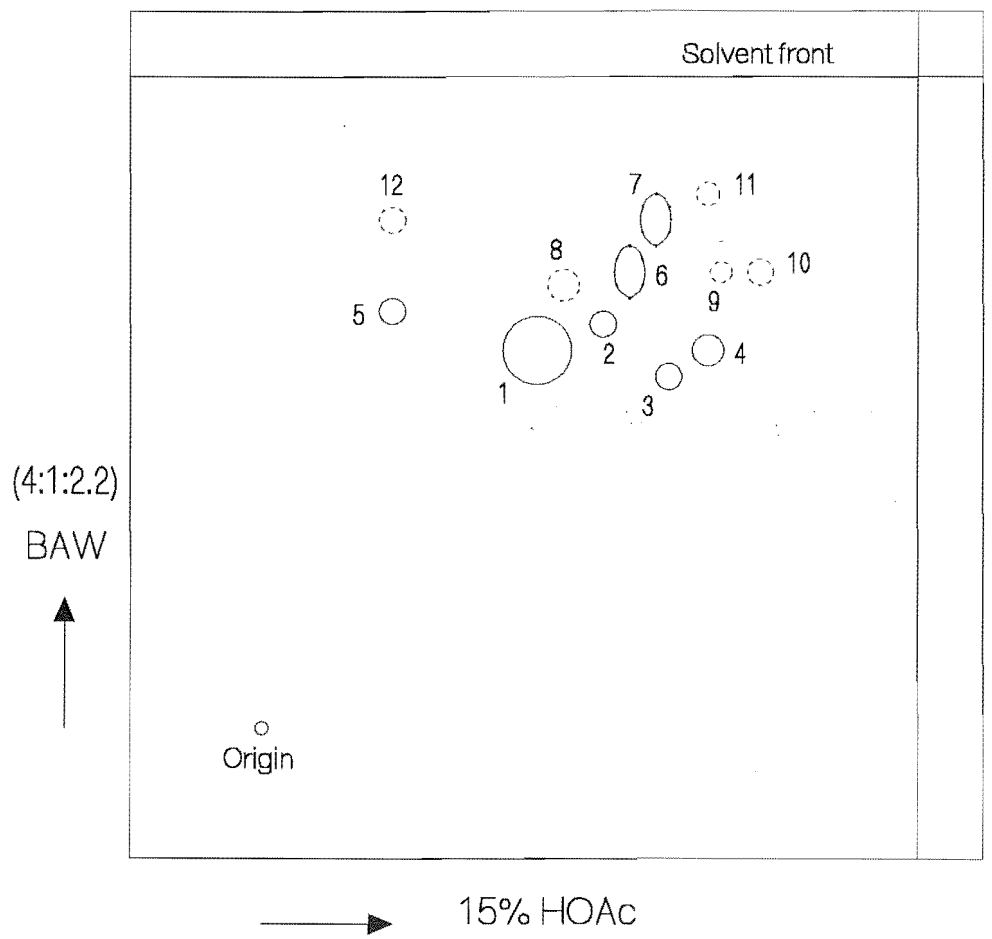


Figure 3.15 Two-dimensional TLC of leaf flavonoids.
The dotted spots represent compounds which were not visible under UV light, but were visible after spraying with NA-reagent. Note that the Rf values, colour and identities (where known) of these spots are given in Table 3.20.

3.4.5 Identification of anthocyanins from leaves

When 2-D TLC's of leaf extracts were developed no anthocyanins were visible, and it was confirmed by analytical HPLC that the leaves of the 29 cultivars tested did not contain anthocyanins.

3.4.6 Identification of flavonoids and phenolic acids from leaves

The initial investigation of leaf flavonoids and phenolic acids by 2-D TLC (Figure 3.15) showed seven compounds (spots 1-7) and after spraying with NA-reagent another five spots appeared (spots 8-12). The chromatographic properties of these compounds are summarised in Table 3.20.

Table 3.20 Chromatographic properties of leaf flavonoids from TLC (from Figure 3.15).

Spot	Rf	Rf	Colour of spot			Tentative identity
no	(a)	(b)	(1)	(2)	(3)	
1	59.0	42.8	purple	yellow	orange	kaempferol-3-rutinoside
2	61.9	52.2	blue	green	blue/green	phenolic
3	54.6	63.4	purple	orange	orange	quercetin-glycoside
4	58.0	68.8	blue	orange	blue	phenolic
5	64.2	20.6	purple	orange	orange	quercetin-3-glucoside
6	67.7	57.8	blue	green	blue	chlorogenic acid
7	76.0	59.7	blue	blue	blue	phenolic
8	68.8	46.3	-	-	orange	flavonoid
9	69.1	69.4	-	-	blue	phenolic
10	69.7	76.9	-	-	orange	flavonoid
11	80.2	68.1	-	-	blue	phenolic
12	78.0	20.6	-	-	blue	phenolic

(a) Rf (x100) BAW

(b) Rf (x100) 15% acetic acid

(1) Colour of spot under UV light

(2) Colour of spot under UV light after exposure to NH_3 fumes

(3) Colour of spot after spraying with NA-reagent, viewed under UV light

Spots 1, 3, and 5 displayed a dull purple colour in UV light, orange or yellow after being exposed to ammonia fumes, and orange after being sprayed with NA-reagent. This indicated that they were all flavonols and, after comparison of Rf values (Appendix 2), it was thought that the probable identities were Km-3-rut, a Qu-gly and Qu-3-glu respectively. The identity of spot 6 was thought to be chlorogenic acid. Other flavonoids (spots 8 and 10) and phenolics (spots 2, 4, 7, 9, 11 and 12) remained unidentified.

The leaf extracts were analysed further by analytical HPLC. A typical HPLC chromatogram of a leaf extract at 280nm is shown in Figure 3.16, and results and possible identifications, after comparison of retention times and spectra of authentic standards, are shown in Table 3.21. The major phenolic acid present was chlorogenic acid (peak e), with moderate amounts of ferulic acid (peak n) and the unknown phenolic, UP1 (peak h). As for flower extracts, moderate amounts of flavonoids were detected, especially the Qu-glycosides, 2a (peak k) and 2b (peak l), and rutin (peak q). (Note that these leaf extracts were run on a new column, so retention times are slightly altered from those shown in tuber and flower chromatograms (Figures 3.3, 3.8, 3.9, 3.11, 3.13) and noted in Appendix 2.)

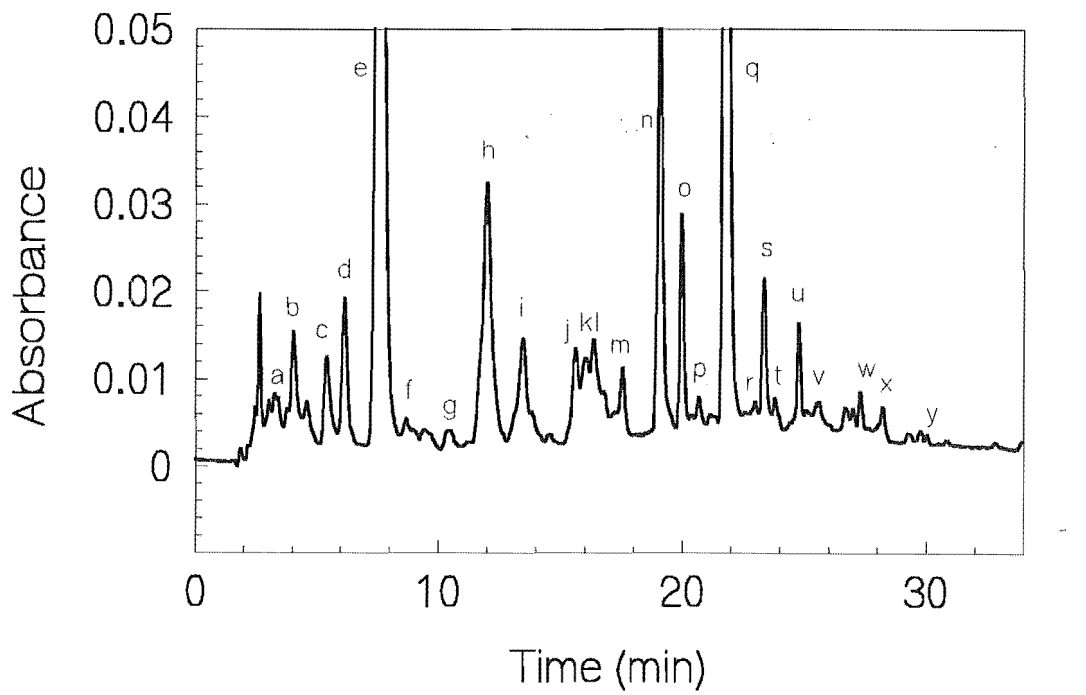


Figure 3.16 Typical HPLC chromatogram of leaf extract at 280nm.
Note that the retention times, spectral peaks and identities of these peaks are given in Table 3.21.

Table 3.21 HPLC data of leaf phenolics (from Figure 3.16) (sh = shoulder).

Peak	Retention time	Spectra	Identity
a	2.8	255	gallic acid
b	4.1	260	protocatechuic acid
c	5.8	280	catechin
d	6.2	255	*p*-hydroxybenzoic acid
e	7.7	300sh, 325	chlorogenic acid
f	9.2	300sh, 320	caffeic acid
g	10.5	280	epicatechin
h	12.0	300sh, 320	unknown phenolic (UP1)
i	13.2	275	syringic acid
j	15.7	300sh, 315	*p*-coumaric acid
k	16.2	255, 350	flavonoid 2a
l	16.8	255, 350	flavonoid 2b
m	17.5	265, 350	unknown flavonoid (UF1)
n	19.2	300sh, 325	ferulic acid
o	19.9	260, 350	quercetin-3-galactoside
p	20.7	320	sinapic acid
q	21.6	255, 350	rutin
r	22.8	260, 350	quercetin-3-glucoside
s	23.2	280, 320sh	eriodictyol
t	23.8	300sh, 325	unknown phenolic (UP3)
u	24.6	270, 350	kaempferol-3-rutinoside
v	25.6	265, 365	myricetin
w	27.2	265, 350	unknown flavonoid (UF2)
x	28.1	280	cinnamic acid
y	29.9	290, 325	naringenin

CHAPTER 3 - Part 2. Analysis of phenolics

3.5 Results - Part 2

Part 2 of these results covers the survey of anthocyanins, flavonoids and phenolic acids in the tubers (skin and flesh), flowers and leaves of *S. tuberosum* cultivars and other *Solanum* species. Comparison of the concentrations of anthocyanins, flavonoids and phenolic acids with enzyme activities in the tubers of five *S. tuberosum* cultivars was carried out, as well as a brief investigation of the effect of disease on the concentrations of these phenolics.

3.5.1 *Solanum tuberosum* cultivars

The characteristics and parentage of *S. tuberosum* cultivars used in this study are shown in Appendix 1 (Tables A1.1 and A1.2). The λ_{max} values of extracts from tubers, flowers and leaves of *S. tuberosum* cultivars were measured to give an indication of the identity of the major anthocyanin present in each sample (Table 3.22). Samples were also analysed by TLC (developed in BAW), and the identity of the spots determined. Samples which contained anthocyanins were hydrolysed in acid and the aglycone fraction analysed by TLC (developed in Forestal) (Table 3.22). These results were used to confirm the identities of the major anthocyanins determined later by HPLC analyses.

Table 3.22 Spectral and chromatographic analysis of the major anthocyanins contained in *Solanum tuberosum* extracts.

Table 3.22a) Skin

Cultivar	Unhydrolysed glycosides				Aglycones		
	λ_{max}	Rf BAW	Colour	Identity	Rf Forestal	Colour	Identity
Arran Victory	538	50.6	purple	anth-1	37.8	purple	Pt
Blue Derwent	538	49.5	purple	anth-1	38.9	purple	Pt
Catriona	-	-			-		
Desirée	510	45.7	pink	Pg-3-r	58.1	purple	Pn
		55.3	pink	Pn-gly	61.9	pink	Pg*
		76.1	pink	anth-3			

continued...

Note anth-1, anth-2, and anth-3 have been identified as Pt-3-(*p*-coumaroyl-rut)-5-glu, Mv-3-(*p*-coumaroyl-rut)-5-glu and Pg-3-(*p*-coumaroyl-rut)-5-glu respectively (Section 3.4.1), similarly flavonoids 2a and 2b have been identified as Qu-gly 2a and Qu-gly 2b (Section 3.4.4).

Table 3.22a) continued...

Cultivar	Unhydrolysed glycosides				Aglycones		
	λ_{max}	Rf BAW	Colour	Identity	Rf Forestal	Colour	Identity
I29	538	49.2	pink	Pg-3-r	60.5	pink	Pg
		76.0	pink	anth-3			
I53	538	50.3	purple	anth-1	37.6	purple	Pt
Kowiniwini	538	49.7	purple	anth-1	38.3	purple	Pt*
		56.0	purple	anth-2	51.7	purple	Mv
Maori Chief	538	49.3	purple	anth-1	37.8	purple	Pt
MoeMoe	538	49.3	purple	anth-1	37.1	purple	Pt
Northern Star	-	-			-		
Old Red	538	48.9	purple	anth-1	38.9	purple	Pt
		55.0	purple	anth-2	53.5	purple	Mv*
O60/1	514	76.9	pink	anth-3	60.1	pink	Pg
Poiwa	538	49.3	purple	anth-1	38.9	purple	Pt
Raupi	538	49.0	purple	anth-1	38.3	purple	Pt
Red Flesh	510	49.9	pink	Pg-3-r	58.2	purple	Pn
		54.7	pink	Pn-gly	61.9	pink	Pg*
		77.6	pink	anth-3			
Red Rascal	514	75.5	pink	anth-3	60.1	pink	Pg
Red Rocks	510	48.3	pink	Pg-3-r	60.4	pink	Pg
		76.5	pink	anth-3			
Rua	-	-			-		
Russet Burbank	-	-			-		
Skerry Blue	538	30.2	purple		36.5	purple	Pt*
		48.8	purple	anth-1	51.0	purple	Mv
Stage II Blue	538	51.1	purple	anth-1	37.3	purple	Pt*
		56.5	purple	anth-2	52.6	purple	Mv
Tekau	532	X			X		
Urenika	538	49.7	purple	anth-1	37.3	purple	Pt
		54.4	purple	anth-2	52.2	purple	Mv*
Whitu	-	-			-		
177/3	-	-			-		
289/3	532	X			X		
1858.21	-	-			-		

Table 3.22b) Flesh

Cultivar	Unhydrolysed glycosides				Aglycones		
	λ_{max}	Rf BAW	Colour	Identity	Rf Forestal	Colour	Identity
Arran Victory	538	50.0	purple	anth-1	X		
I53	538	50.0	purple	anth-1	36.5	purple	Pt
Red Flesh	510	76.8	pink	anth-3	61.9	pink	Pg
Skerry Blue	538	48.3	purple	anth-1	X		
Stage II Blue	538	50.0	purple	anth-1	X		
		55.0	purple	anth-2			
Urenika	538	48.3	purple	anth-1	36.7	purple	Pt
		55.0	purple	anth-2	53.6	purple	Mv*
1858.21	532	X			X		

Table 3.22c) Flowers

Cultivar	Unhydrolysed glycosides			
	λ_{max}	Rf BAW	Colour	Identity
Catriona	532	57.3	purple	anth-2
Desirée	528	53.4	pink	Pn-gly
I29	528	X		
I53	528	X		
Kowiniwini	532	48.3	purple	anth-1
		54.8	purple	anth-2
MoeMoe	538	48.8	purple	anth-1
M10	528	X		
Ngaoutiouti	538	48.4	purple	anth-1
O60/1	528	57.2	purple	anth-2
Poiwa	538	48.4	purple	anth-1
Raupi	534	48.6	purple	anth-1
		55.0	purple	anth-2
Red Flesh	524	54.2	pink	Pn-gly

continued...

Table 3.22c) continued...

Cultivar	Unhydrolysed glycosides			
	λ_{max}	Rf BAW	Colour	Identity
Red Rascal	528	42.6	pink	
		51.6	pink	Pn-gly
Red Rocks	528	X		
Rua	528	X		
Russet Burbank	528	X		
Skerry Blue	538	49.7	purple	anth-1
Stage II Blue	532	X		
Tekau	532	X		
Urenika	538	48.8	purple	anth-1
177/3	534	48.2	purple	anth-1
289/3	532	49.1	purple	anth-1
1858.21	528	50.1	purple	anth-1

* = in cultivars with two aglycone spots * indicates the strongest spot

X = sample not run on TLC (not enough anthocyanin present)

- = no anthocyanin present

Mv = malvidin

Pg = pelargonidin

Pt = petunidin

anth-1 = petunidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside

anth-2 = malvidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside

anth-3 = pelargonidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside

Pg-3-r = pelargonidin-3-rutinoside

Pn-gly = peonidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside

These extracts from *S. tuberosum* cultivars were subsequently analysed by analytical HPLC, and the identities and concentrations of phenolic acids, flavonoids and anthocyanins determined and calculated by comparison with authentic standards (Chapter 3.4 and Appendix 3). The identity of the major anthocyanin(s) present in each sample

agreed with that determined by TLC (Table 3.22). The total concentration of phenolic acids, flavonoids and anthocyanins, and percentage of each class for each cultivar is given in Table 3.23.

Table 3.23 Concentrations and percentages of phenolic acids, flavonoids and anthocyanins found in *Solanum tuberosum* cultivars.
(See Appendix 1, Table A1.2 for tuber and flower colours)

Cultivar	Plant tissue	Phenolic acids	Flavonoids	Anthocyanins	Ratio (%)#
		µg/gFW			
Arran Victory	* skin	2785.3	289.6	1700.2	58.3/6.1/35.6
purple skin	* flesh	638.6	27.1	106.9	82.7/3.5/13.8
white flesh	** skin	3879.1	353.9	2386.9	58.6/5.3/36.1
	** flesh	345.6	29.9	78.2	76.1/6.6/17.2
	* flowers[1]				
	* leaves	693.0	1710.7	0	28.8/71.2/0
Blue Derwent	* skin	4393.0	289.9	2485.8	61.3/4.1/34.7
purple skin	* flesh	292.1	9.2	10.8	93.6/2.9/3.5
white flesh	** skin	4809.9	242.7	3089.9	59.1/3.0/37.9
	** flesh	255.4	7.4	103.7	69.7/2.0/28.3
	* flowers[1]				
	* leaves	368.8	637.5	0	36.6/63.4/0
Catriona	* skin	1784.1	207.0	0	89.6/10.4/0
white skin	* flesh	139.7	0	0	100/0/0
white flesh	** skin	3220.0	331.3	238.7	85.0/8.7/6.3
	** flesh	115.7	4.7	0	96.1/3.9/0
	* flowers	902.4	1554.6	1006.6	26.1/44.9/29.1
	* leaves	823.3	1069.0	0	43.5/56.5/0
Desirée	* skin	2292.3	336.9	712.5	68.6/10.1/21.3
red skin	* flesh	337.5	15.3	0	95.7/4.3/0
white flesh	** skin	2923.3	392.3	1445.4	61.4/8.2/30.4
	** flesh	273.5	10.2	0	96.4/3.6/0
	* flowers	732.7	2127.5	171.5	24.2/70.2/5.7
	* leaves	311.0	725.2	0	30.0/70.0/0

continued...

Table 3.23 continued...

Cultivar	Plant tissue	Phenolic acids	Flavonoids	Anthocyanins	Ratio (%)#
		µg/gFW			
I29	* skin	3432.1	255.5	194.5	88.4/6.6/5.0
red skin	* flesh	201.9	31.8	0	86.4/13.6/0
white flesh	** skin	3256.6	373.3	448.2	79.9/9.2/11.0
	** flesh	480.5	27.7	0	94.5/5.5/0
	* flowers	910.9	1933.4	17.3	31.8/67.6/0.6
	* leaves	1142.5	1316.3	0	46.5/53.5/0
I53	* skin	2338.4	261.3	719.6	70.4/7.9/21.7
purple skin	* flesh	447.0	29.3	463.6	47.6/3.1/49.3
purple flesh	** skin	5150.7	548.4	3480.9	56.1/6.0/37.9
	** flesh	458.3	20.8	522.3	45.8/2.1/52.2
	* flowers	698.6	2558.7	8.3	21.4/78.4/0.3
	* leaves	602.7	1005.8	0	37.5/62.5/0
Kowiniwini	* skin	2036.0	239.7	1986.4	47.8/5.6/46.6
purple skin	* flesh	185.7	4.6	8.9	93.2/2.3/4.5
white flesh	** skin	2141.1	219.2	2016.0	48.9/5.0/46.1
	** flesh	171.0	15.6	43.8	74.2/6.8/19.0
	* flowers[1]				
	* leaves	726.9	1334.1	0	35.3/64.7/0
Maori Chief	* skin	1609.3	279.0	806.3	59.7/10.4/29.9
purple skin	* flesh	191.5	7.0	24.6	85.8/3.1/11.0
white flesh	** skin	2050.4	310.8	656.1	68.0/10.3/21.7
	** flesh	204.8	6.7	0	96.8/3.2/0
	* flowers[1]				
	* leaves	260.6	551.4	0	32.1/67.9/0
Moe Moe	* skin	2386.5	287.8	824.7	68.2/8.2/23.6
purple skin	* flesh	168.7	7.5	8.3	91.4/4.1/4.5
white flesh	** skin	2989.4	337.2	691.7	74.4/8.4/17.2
	** flesh	149.3	7.7	18.5	85.1/4.4/10.5
	* flowers	1081.1	1834.4	697.3	29.9/50.8/19.3
	* leaves	566.6	1038.0	0	35.3/64.7/0

continued...

Table 3.23 continued...

Cultivar	Plant tissue	Phenolic acids	Flavonoids	Anthocyanins	Ratio (%)#
		µg/gFW			
M10	* skin	1297.6	165.4	0	88.7/11.3/0
white skin	* flesh	289.3	4.0	0	98.6/1.4/0
white flesh	* flowers	549.6	1094.3	0	33.4/66.6/0
	* leaves	161.5	485.8	0	24.9/75.1/0
Ngaoutiouti	* skin	1733.0	110.2	431.1	76.2/4.8/19.0
purple skin	* flesh	156.1	4.9	38.4	78.3/2.5/19.3
white flesh	** skin	2932.9	227.3	1083.7	69.1/5.4/25.5
	** flesh	150.2	10.7	30.2	78.6/5.6/15.8
	* flowers	1036.8	1746.5	475.0	31.8/53.6/14.6
	* leaves	1116.6	1959.8	0	36.3/63.7/0
Northern Star	* skin	1497.5	169.6	15.4	89.0/10.1/0.9
white skin	* flesh	105.6	2.7	0	97.5/2.5/0
white flesh	** skin	2908.9	253.0	162.5	87.5/7.6/4.9
	** flesh	122.5	11.8	0	91.2/8.8/0
	* flowers[1]				
	* leaves	482.3	894.4	0	35.0/65.0/0
Old Red	* skin	2360.7	234.5	1409.4	58.9/5.9/35.2
purple skin	* flesh	160.8	5.1	0	96.9/3.1/0
white flesh	** skin	2720.6	281.5	2591.4	48.6/5.0/46.3
	** flesh	194.9	0	14.6	93.0/0/7.0
	* flowers[1]				
	* leaves	444.8	1423.1	0	23.8/76.2/0
O60/1	* skin	2309.8	324.6	220.0	80.9/11.4/7.7
red skin	* flesh	337.2	25.7	0	92.9/7.1/0
white flesh	** skin	3311.5	479.6	772.8	72.6/10.5/16.9
	** flesh	283.3	12.2	0	95.9/4.1/0
	* flowers	898.1	1341.0	1404.3	24.7/36.8/38.5
	* leaves	845.3	927.9	0	47.7/52.3/0

continued...

Table 3.23 continued...

Cultivar	Plant tissue	Phenolic acids	Flavonoids	Anthocyanins	Ratio (%)#
		µg/gFW			
Poiwa	* skin	1933.8	158.8	396.4	77.7/6.4/15.9
purple skin	* flesh	147.6	7.2	0	95.3/4.7/0
white flesh	** skin	1791.9	193.7	246.3	80.3/8.7/11.0
	** flesh	119.2	5.5	0	95.6/4.4/0
	* flowers	1014.4	1812.8	688.1	28.9/51.6/19.6
	* leaves	454.9	770.2	0	37.1/62.9/0
Raupi	* skin	975.8	227.1	369.4	62.1/14.4/23.5
purple skin	* flesh	126.0	1.9	0	98.5/1.5/0
white flesh	** skin	2030.5	254.2	997.7	61.9/7.7/30.4
	** flesh	120.3	9.5	11.9	84.9/6.7/8.4
	* flowers	1127.9	2256.4	1592.0	22.7/45.3/32.0
	* leaves	880.4	1417.0	0	38.3/61.7/0
Red Flesh	* skin	3239.1	335.3	1953.6	58.6/6.1/35.3
red skin	* flesh	736.1	30.9	98.6	85.0/3.6/11.4
red flesh	** skin	3889.7	492.9	3325.2	50.5/6.4/43.1
	** flesh	628.6	24.0	114.8	81.9/3.1/15.0
	* flowers	895.1	1197.7	59.2	41.6/55.7/2.8
	* leaves	998.6	2126.9	0	32.0/68.0/0
Red Rascal	* skin	2273.2	220.9	605.0	73.4/7.1/19.5
red skin	* flesh	345.3	17.6	0	95.2/4.8/0
white flesh	** skin	2880.6	327.7	1091.5	67.0/7.6/25.4
	** flesh	235.8	16.2	0	93.6/6.4/0
	* flowers	750.4	2292.7	200.5	23.1/70.7/6.2
	* leaves	1596.0	2010.3	0	44.3/55.7/0
Red Rocks	* skin	2169.8	216.5	262.9	81.9/8.2/9.9
red skin	* flesh	255.4	10.2	0	96.2/3.8/0
white flesh	** skin	2514.8	161.6	204.6	87.3/5.6/7.1
	** flesh	111.3	8.9	0	92.6/7.4/0
	* flowers	981.4	2854.5	31.1	25.4/73.8/0.8
	* leaves	797.6	1339.2	0	37.3/62.7/0

continued...

Table 3.23 continued...

Cultivar	Plant tissue	Phenolic acids	Flavonoids	Anthocyanins	Ratio (%)#
		µg/gFW			
Russet Burbank	* skin	1415.2	206.6	0	87.3/12.7/0
white skin	* flesh	243.1	17.1	0	93.4/6.6/0
white flesh	** skin	2044.9	246.9	0	89.2/10.8/0
	** flesh	129.7	18.2	0	87.7/12.3/0
	* flowers	813.6	2222.4	40.6	26.4/72.2/1.3
	* leaves	216.2	683.7	0	24.0/76.0/0
Skerry Blue	* skin	2716.4	319.1	1116.2	65.4/7.7/26.9
purple skin	* flesh	288.0	17.5	89.3	72.9/4.4/22.6
white flesh	** skin	3561.5	315.8	1579.2	65.3/5.8/28.9
	** flesh	173.1	9.0	33.9	80.1/4.2/15.7
	* flowers[1]				
	* leaves	996.8	1993.8	0	33.3/66.7/0
Stage II Blue	* skin	3036.7	252.1	2643.4	51.2/4.2/44.6
purple skin	* flesh	396.4	10.0	29.5	90.9/2.3/6.8
purple flesh	** skin	6239.7	453.6	7446.6	44.1/3.2/52.7
	** flesh	516.0	17.5	218.5	68.6/2.3/29.1
	* flowers	706.2	1808.2	9.5	28.0/71.6/0.4
	* leaves	1228.3	2036.3	0	37.6/62.4/0
Tekau	* skin	1230.2	148.2	26.1	87.6/10.6/1.9
white skin	* flesh	168.3	11.7	0	93.5/6.5/0
white flesh	** skin	2069.3	194.5	81.8	88.2/8.3/3.5
	** flesh	145.1	8.8	0	94.3/5.7/0
	* flowers	824.5	2979.4	33.5	21.5/77.6/0.9
	* leaves	1211.0	1092.8	0	52.6/47.4/0
Urenika	* skin	3828.7	146.4	4376.4	45.8/1.8/52.4
purple skin	* flesh	1221.0	41.3	2320.3	34.1/1.2/64.8
purple flesh	** skin	5344.4	162.5	5778.8	47.4/1.4/51.2
	** flesh	1229.0	44.2	1351.6	46.8/1.7/51.5
	* flowers	767.2	1217.1	189.8	35.3/56.0/8.7
	* leaves	403.7	693.3	0	36.8/63.2/0

continued...

Table 3.23 continued...

Cultivar	Plant tissue	Phenolic acids	Flavonoids	Anthocyanins	Ratio (%)#
		µg/gFW			
Whitu	* skin	1044.6	149.7	16.6	86.3/12.4/1.4
white skin	* flesh	188.5	26.3	0	87.7/12.2/0
white flesh	* flowers[1]				
	* leaves	783.0	1641.1	0	32.3/67.7/0
177/3	* skin	935.1	158.3	0	85.5/14.5/0
white skin	* flesh	72.6	9.3	0	88.6/11.4/0
white flesh	** skin	1679.7	210.9	0	88.8/11.2/0
	** flesh	77.5	12.1	0	86.5/13.5/0
	* flowers	316.5	1896.7	707.4	10.8/64.9/24.2
	* leaves	1416.0	2164.6	0	39.5/60.5/0
289/3	* skin	1802.3	271.1	24.3	85.9/12.9/1.2
white skin	* flesh	269.8	20.7	0	92.9/7.1/0
white flesh	** skin	2512.0	293.6	307.3	80.7/9.4/9.9
	** flesh	154.8	18.0	0	89.6/10.4/0
	* flowers	698.6	2033.3	1678.2	15.8/46.1/38.1
	* leaves	570.4	991.3	0	36.5/63.5/0
1858.21	* skin	817.1	118.3	0	87.4/12.6/0
white skin	* flesh	142.4	8.6	0	94.3/5.7/0
white flesh	** skin	1543.8	221.2	51.1	85.0/12.2/2.8
	** flesh	140.1	16.2	0	89.6/10.4/0
	* flowers	689.3	2345.9	111.7	21.9/74.5/3.5
	* leaves	512.1	689.3	0	42.6/57.4/0

The colour of the skin and flesh in italics represents the colour classes in which tubers were grouped in for analyses.

\# ratio of phenolic acids : flavonoids (excluding anthocyanins) : anthocyanins

* 1992/93 season

** 1993/94 season

1 produced too few flowers for analysis or did not flower

Tubers (skin and flesh) were found to have a very high percentage of phenolic acids (an average of 79.1%) and a relatively low percentage of flavonoids (excluding anthocyanins) (an average of 8.6% flavonoids), whereas the flowers had a high percentage of flavonoids (an average of 61.6%) and a much lower percentage of phenolic acids (an average of 25.2%) (Table 3.23). The tubers and flowers had varying concentrations of anthocyanins, but in tubers the percentage concentration of flavonoid remained constant and the percentage of phenolic acids was related to the anthocyanin concentration. By contrast, in flowers the percentage of phenolics remained relatively constant and the flavonoid percentage altered with the anthocyanin percentage. Leaves contained, on average, 36.4% phenolics acids, 63.6% flavonoids and no anthocyanins (Table 3.23).

3.5.1.1 Total concentration of phenolic acids, flavonoids and anthocyanins in tubers

The tubers of *S. tuberosum* cultivars were grouped according to tuber colour (for skin colours, white, purple and red, and for flesh colours, white skin with white flesh, coloured skin with white flesh, purple skin with purple flesh, and red skin with red flesh) (Table 3.23). This grouping of tubers into colour groups was carried out to establish whether or not there were any significant differences in phenolic concentrations related to the colour of the tuber, and to determine where the biochemical pathways differed in tubers which produced anthocyanin and compared with those which did not. The concentrations of total phenolic acids, flavonoids and anthocyanins were averaged for each colour group and are shown in Figure 3.17. Because of the wide range of concentrations present, these are shown on a log scale and, whilst this enables the thousand-fold difference to be viewed easily on the same axis, the significant differences between tuber colours of compounds at high concentrations are often not visible. Data in the figures are presented as averages of the colour groups, whilst the ranges presented in the text are quoted from the data presented in Appendix 3 and represent the range of concentrations found in the different cultivars (Tables A3.1, A3.2 and A3.3).

Skin

Depending on the cultivar, the skin of tubers contained between 2000-5000μg/gFW phenolic acids, 200-300μg/gFW flavonoids and 0-7000μg/gFW anthocyanins (Figure 3.17a). Purple and red skinned tubers contained almost double the concentration of phenolic acids of white skinned tubers. There was also a smaller but significant difference ($p<0.05$) in the flavonoid concentration between these classes, with coloured tubers containing higher concentrations of flavonoids than white tubers. Not surprisingly there was a huge increase in anthocyanin concentration from white to coloured tubers (Figure 3.17a).

There was also a difference in phenolic acid, flavonoid and anthocyanin concentrations between the 1992/93 and 1993/94 seasons, where the 1993/94 season had significantly higher concentrations of all these three classes in the skin of tubers (Figure 3.17a). However, for each season the difference between white and coloured skinned tubers showed the same general pattern.

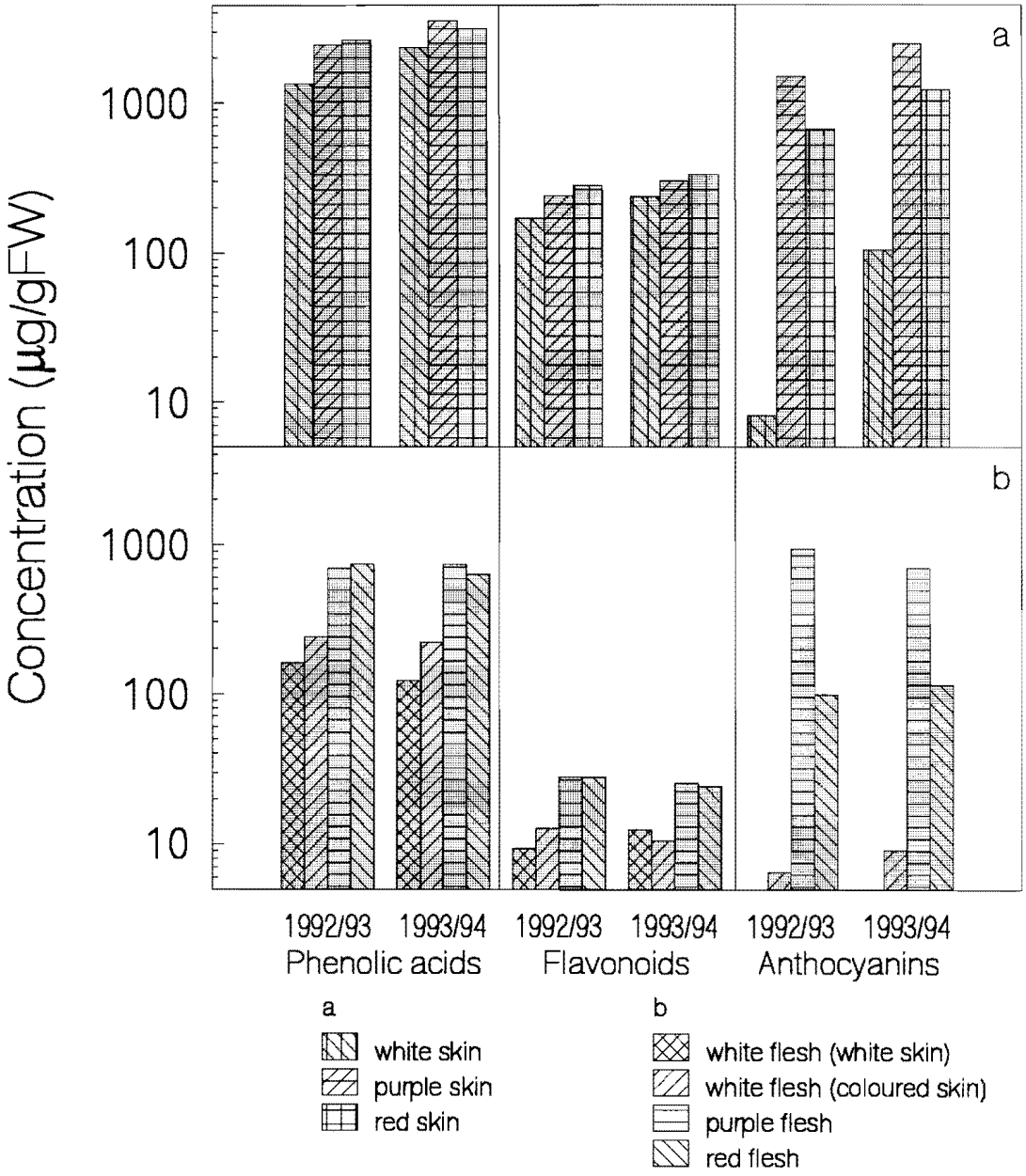


Figure 3.17 Total concentration of phenolics in a) skin and b) flesh of white, purple and red *Solanum tuberosum* cultivars.

Flesh

Tuber flesh contained much lower concentrations of phenolic acids, flavonoids and anthocyanins than the skin (per gFW); often an order of magnitude lower. Depending on the cultivar, the concentration in the flesh of phenolic acids was between 100-600 μg/gFW, of flavonoids was between 0-30μg/gFW and of anthocyanins was between 0-2000μg/gFW (Figure 3.17b). In contrast to the values for the skin, there was virtually no difference between the 1992/93 and 1993/94 seasons for concentrations of phenolic acids, flavonoids or anthocyanins in the flesh.

The flesh of white fleshed tubers with coloured skin had a significantly ($p<0.05$) higher concentration of phenolic acids than the flesh of white fleshed tubers with white skin (Figure 3.17b). Tubers with coloured flesh (purple or red) had three to four times the concentration of phenolic acids in the flesh than white fleshed tubers, however there was no difference in phenolic acid concentration between purple or red flesh (Figure 3.17b). The flavonoid concentration of coloured fleshed tubers was twice that of white fleshed tubers, but there was no difference between purple and red flesh, or between white flesh with or without coloured skin. Anthocyanin concentration was highest in purple fleshed tubers, followed by red fleshed and then white fleshed tubers with coloured skin, with ten-fold differences between each class. The tubers with white flesh and skin contained no anthocyanin in the flesh.

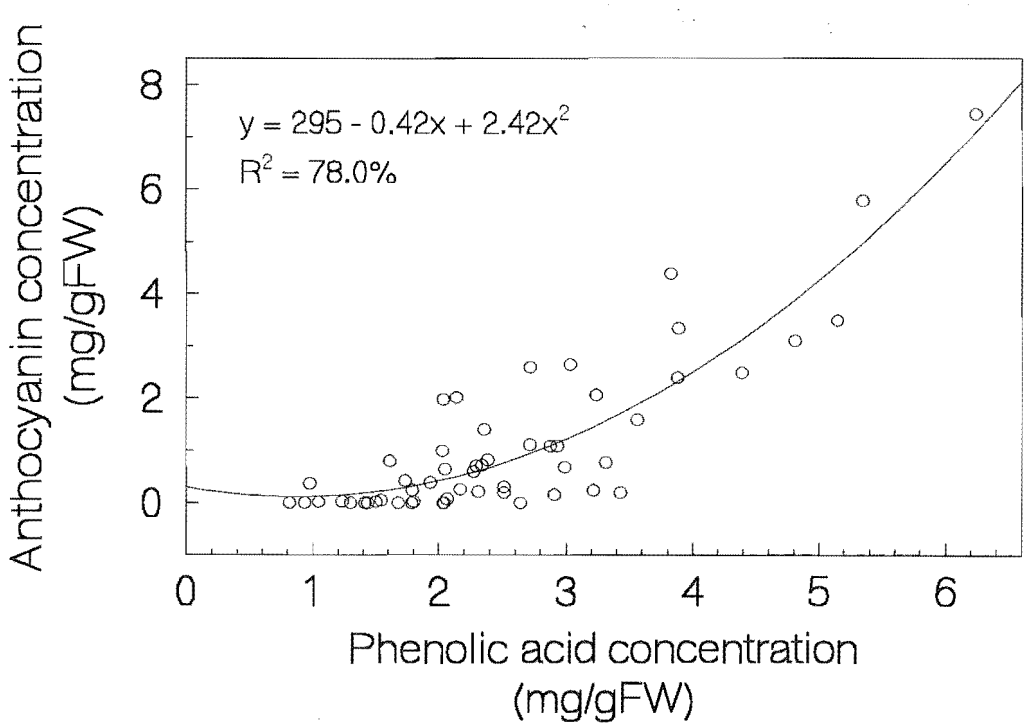


Figure 3.18 Correlation between anthocyanin and phenolic acid concentrations in tubers.

Regression analysis was carried out to determine if there was a correlation between the phenolic acids, flavonoids and anthocyanin concentrations of tuber skin. There were slight positive correlations between anthocyanin and flavonoid concentration (R^2= 23.0%) and between flavonoid and phenolic acid concentration (R^2 = 38.9%). There was a good positive correlation between the anthocyanin and phenolic acid concentrations (R^2 = 78.0%) with more coloured tubers containing higher concentrations of phenolic acids (Figure 3.18). There was also a good positive correlation between anthocyanin and chlorogenic acid concentration (R^2 = 75.9%), which was expected as chlorogenic acid accounted for 60-90% of the total phenolic acid concentration. Therefore, the concentration of anthocyanins was related to the concentration of phenolic acids, but not to the flavonoid concentration.

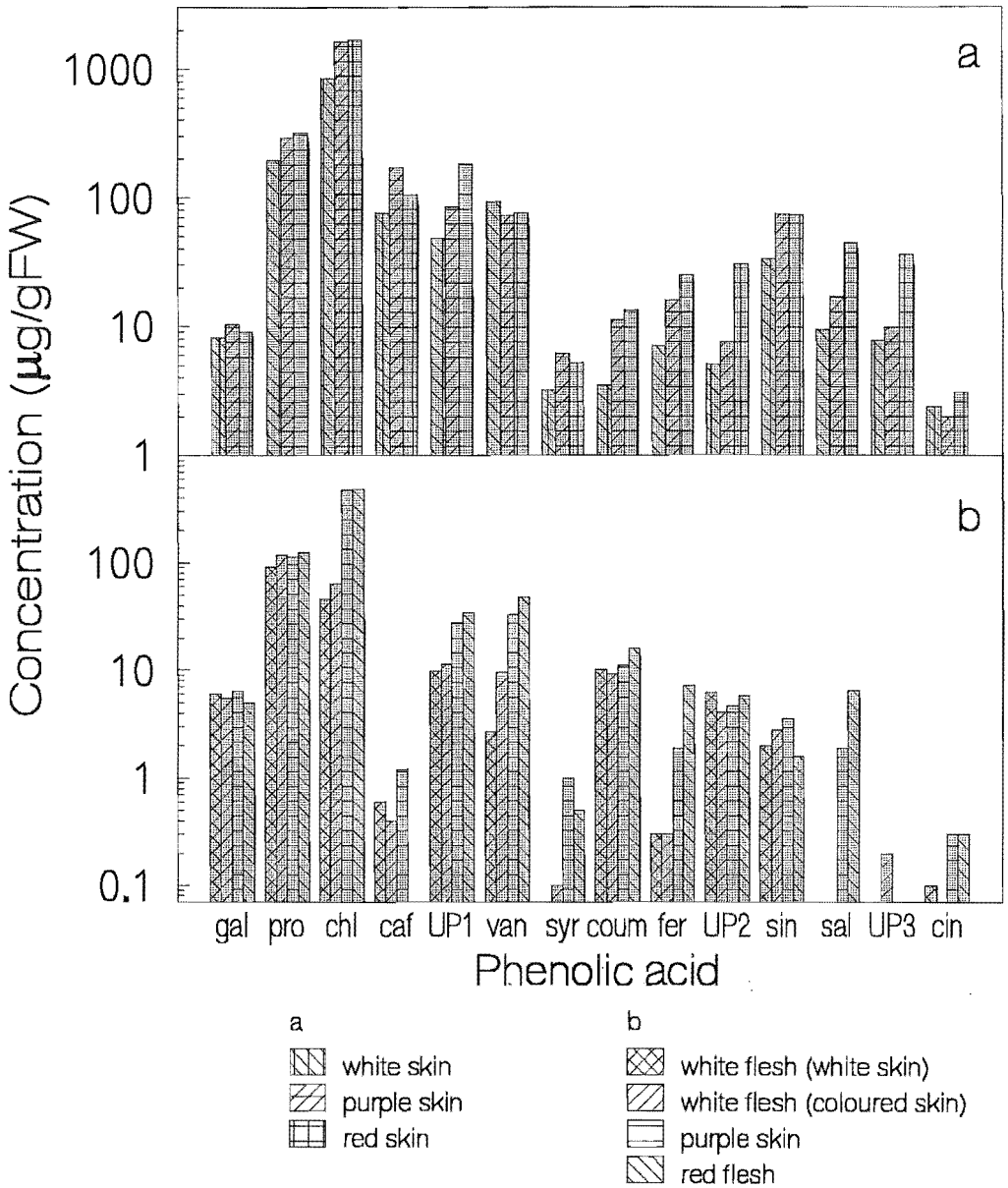
3.5.1.2 Concentrations of individual phenolic acids, flavonoids and anthocyanins in tubers

The average concentrations of individual phenolic acids, flavonoids and anthocyanins using the different colour groups of tubers are shown in Figures 3.19, 3.20 and 3.21. Although the concentration of all the classes of phenolics were higher in the 1993/94 season than the 1992/93 season, the pattern of the individual compounds was similar, therefore only the data for the 1992/93 season are presented in these figures. Data for both seasons are presented in Appendix 3 (Tables A3.4, A3.5 and A3.6).

Phenolic acids

Skin. Tuber skin had high concentrations of chlorogenic acid (1000-4000μg/gFW), with moderate amounts of protocatechuic acid (100-400μg/gFW), caffeic acid (40-500 μg/gFW), UP1 (30-400μg/gFW), vanillic acid (20-200μg/gFW) and sinapic acid (20-250μg/gFW), and lower concentrations of gallic acid, syringic acid, *p*-coumaric acid, ferulic acid, UP2, salicylic acid, UP3 and cinnamic acid (all 0-30μg/gFW) with the concentrations of these depending on the cultivar (Appendix 3, Table A3.1). The average differences in the concentrations of these phenolic acids between the skins of white, purple and red tubers is shown in Figure 3.19a.

Flesh. As previously mentioned, the concentration of phenolic acids was much lower in the flesh than the skin (Section 3.5.1.1). Chlorogenic acid (30-900μg/gFW) and protocatechuic acid (50-200μg/gFW) were present in the highest concentrations, with UP1, vanillic acid and *p*-coumaric acid present in moderate amounts (5-40μg/gFW), and the other phenolic acids present in very small concentrations (0-10μg/gFW) with the concentrations of these phenolic acids depending on the cultivar (Appendix 3, Table A3.1).



gal = gallic acid

pro = protocatechuic acid

chl = chlorogenic acid

caf = caffeic acid

UP1 = unidentified phenolic acid 1

van = vanillic acid

syr = syringic acid

coum = *p*-coumaric acid

fer = ferulic acid

UP2 = unidentified phenolic acid 2

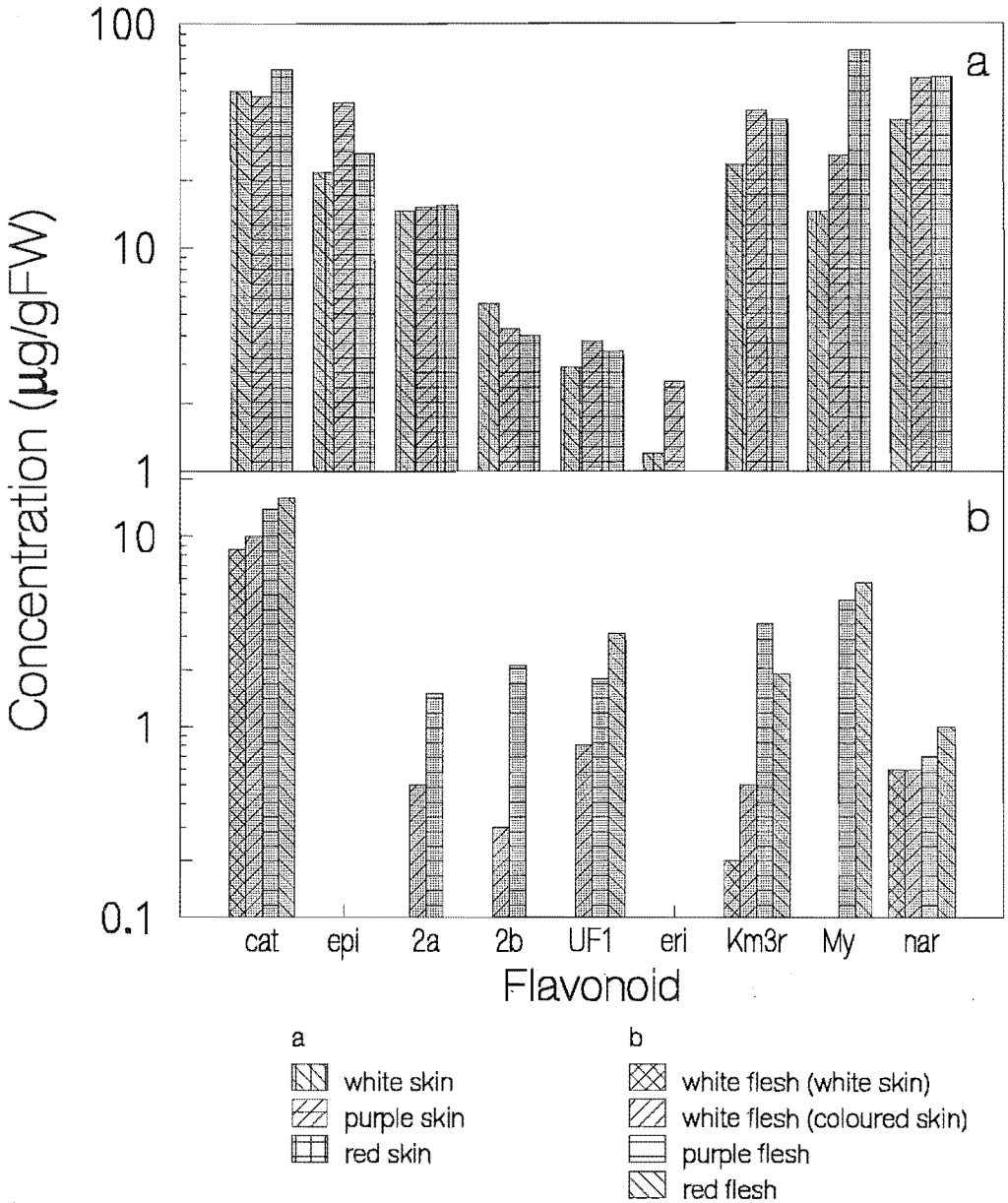
sin = sinapic acid

sal = salicylic acid

UP3 = unidentified phenolic acid 3

cin = cinnamic acid

Figure 3.19 Phenolic acid concentrations in a) skin and b) flesh of different coloured *Solanum tuberosum* cultivars.



cat = catechin

epi = epicatechin

2a = quercetin-glycoside 2a (Qu-gly 2a)

2b = quercetin-glycoside 2b (Qu-gly 2b)

UF1 = unidentified flavonoid 1

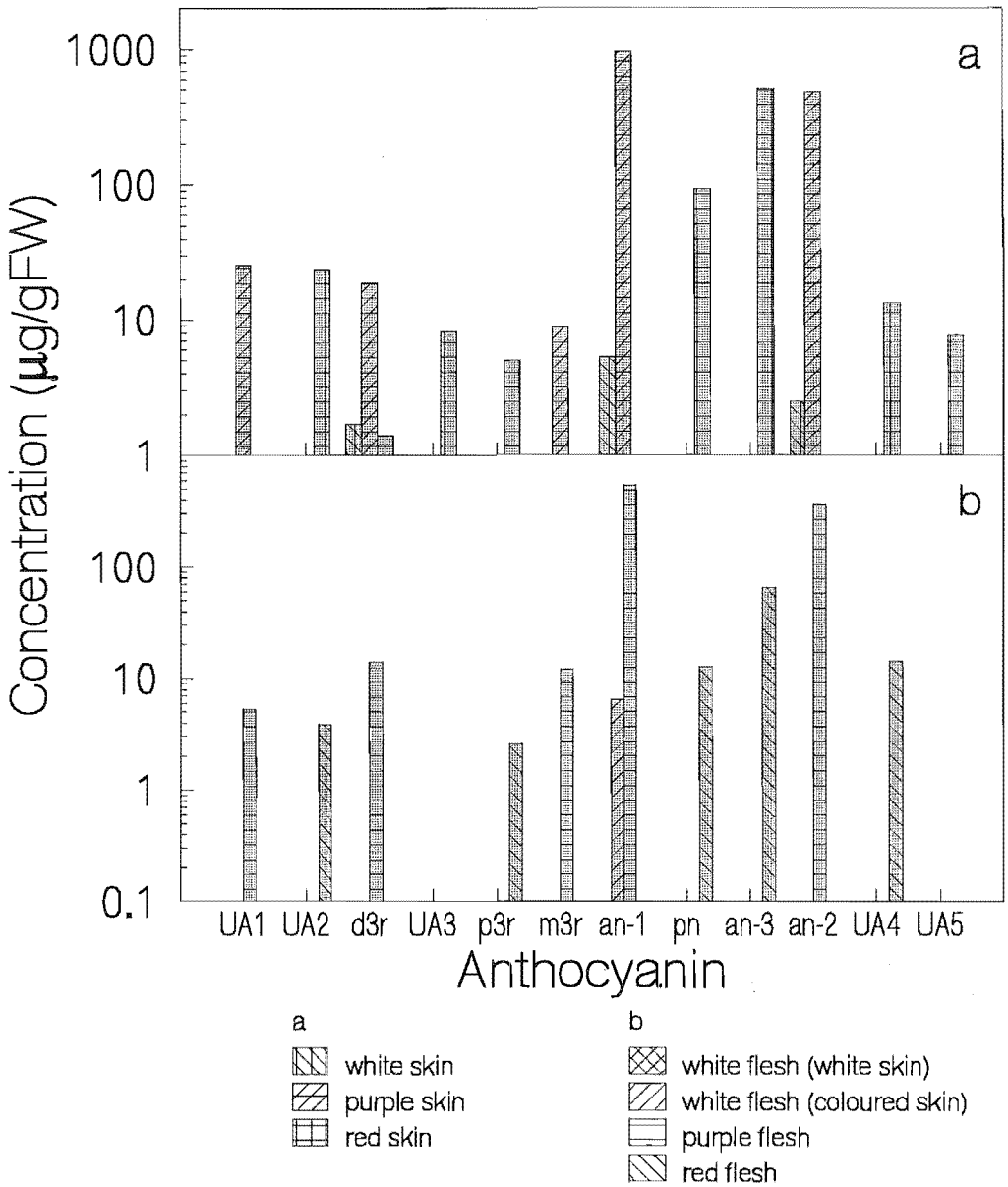
eri = eriodictyol

Km3r = kaempferol-3-rutinoside (Km-3-rut)

My = myricetin

nar = naringenin

Figure 3.20 Flavonoid concentrations in a) skin and b) flesh of different coloured *Solanum tuberosum* cultivars.



UA1 = unidentified anthocyanin 1

UA2 = unidentified anthocyanin 2

d3r = delphinidin-3-rutinoside (Dp-3-rut)

UA3 = unidentified anthocyanin 3

p3r = pelargonidin-3-rutinoside (Pg-3-rut)

m3r = malvidin-3-rutinoside (Mv-3-rut)

an-1 = petunidin-3-(*p*-coumaroyl-rutinoside) -5-glucoside (anth-1)

pn = peonidin-3-(*p*-coumaroyl- rutinoside)-5-glucoside

an-3 = pelargonidin-3-(*p*-coumaroyl-rutinoside) -5-glucoside (anth-3)

an-2 = malvidin-3-(*p*-coumaroyl-rutinoside) -5-glucoside (anth-2)

UA4 = unidentified anthocyanin 4

UA5 = unidentified anthocyanin 5

Figure 3.21 Anthocyanin concentrations in a) skin and b) flesh of different coloured *Solanum tuberosum* cultivars.

The concentrations of chlorogenic acid, UP1, vanillic acid, ferulic acid and salicylic acid were significantly higher in tubers with coloured flesh than white flesh. There was no significant difference of individual phenolic acid concentrations between white flesh with white or coloured skin, or between red and purple flesh (Figure 3.19b).

Flavonoids

Skin and flesh. Flavonoids that showed the highest concentrations in the skin were catechin, epicatechin, eriodictyol, Km-3-rut and naringenin (with concentrations between 10-150μg/gFW), whilst Qu-gly 2a, Qu-gly 2b, UF1 and My had slightly lower concentrations (between 0-40μg/gFW), and UF2 and rutin, and were either not detected, or were present only at very low concentrations (0-5μg/gFW) (Appendix 3, Table A3.2). Catechin was the only flavonoid found consistently in almost all flesh extracts (0-25 μg/gFW). Epicatechin was found in skin extracts, but none was detected in flesh samples.

There was little or no difference in the concentrations of the various flavonoids between white, purple or red skinned tubers; or tubers with white or coloured flesh, however red skinned tubers had a significantly higher concentration of Km-3-rut than did white or purple skinned tubers (Figure 3.20a and b).

Anthocyanins

Skin and flesh. As expected, the white skinned, white fleshed tubers contained very little or no anthocyanin. Tubers with purple skin and/or flesh contained high concentrations of Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1) and Mv-3-(*p*-coumaroyl-rut)-5-glu (anth-2), with lower concentrations of UA1, Dp-3-rut and Mv-3-rut; whereas the red skinned and fleshed tubers contained high concentrations of Pg-3-(*p*-coumaroyl-rut)-5-glu (anth-3) and Pn-3-(*p*-coumaroyl-rut)-5-glu, and lower concentrations of Pg-3-rut and other unknown anthocyanins (Figure 3.21a and b, Appendix 3, Table A3.3).

3.5.1.3 Total concentrations of phenolic acids, flavonoids and anthocyanins in flowers

Flowers contained lower concentrations of phenolic acids (400-1100μg/gFW), higher concentrations of flavonoids (1000-3000μg/gFW) and generally lower concentrations of anthocyanins (0-1600μg/gFW) than did tuber skins, with the concentrations of the individual phenolics depending on the cultivar (Appendix 3). The pattern of flavonoids found in flowers appeared to fall into two categories:-

category 1 - flowers which contained high concentrations of Qu-gly 2a (400-1500μg/gFW), Qu-gly 2b (100-800μg/gFW) and rutin (400-1200μg/gFW), so that the ratio of ([2a] + [2b]) / [rutin] was over one (usually between one and four),

category 2 - flowers which contained very low concentrations of Qu-gly 2a and 2b (20-40μg/gFW) and very high concentrations of rutin (1100-3000μg/gFW), depending on the cultivar, so that the ratio of ([2a] + [2b]) / [rutin] was between 0.02 and 0.05.

Most of the cultivars fell into the first category, with only Catriona, O60/1, Russet Burbank, Tekau, Whitu and 177/3 falling into the second category. Data are presented separately for these two categories in Figures 3.22, 3.23, 3.24 and 3.25, for the total and individual concentrations of phenolics.

The total concentration of phenolic acids, flavonoids and anthocyanins in flowers is shown in Figure 3.22. There was no significant difference between the two categories of flower for any of these phenolic groups; most surprisingly the flavonoids showed no difference in total concentration between the two categories despite the huge difference in individual flavonoid concentrations.

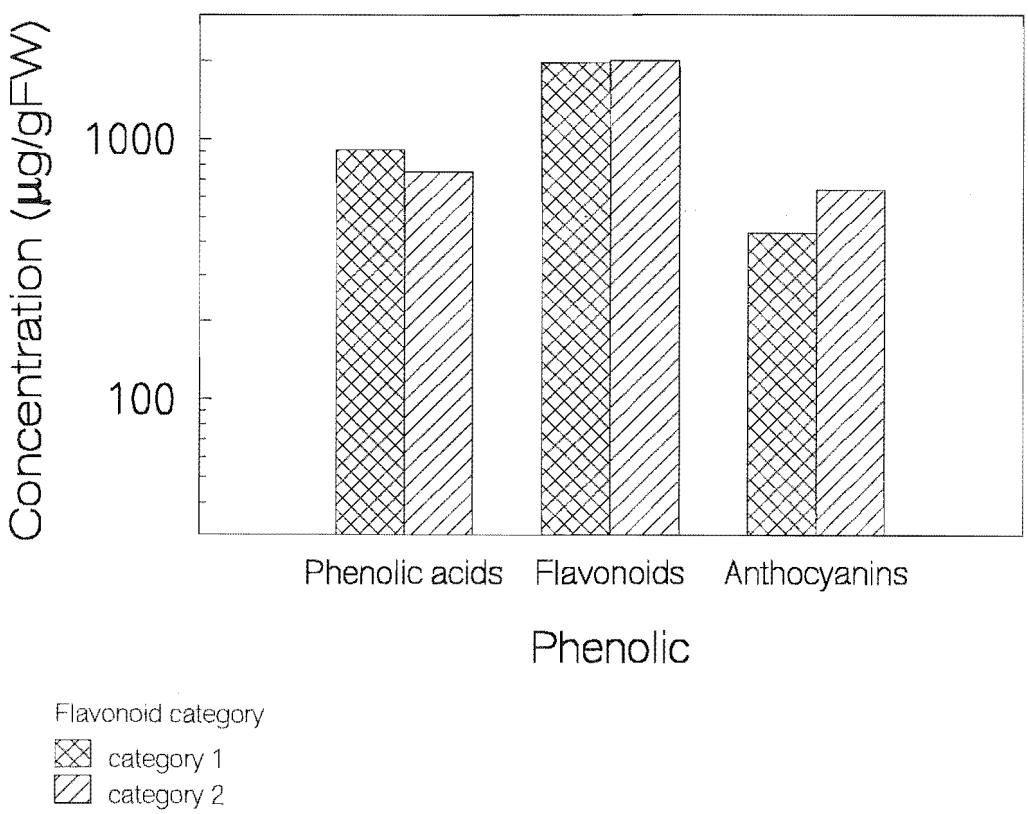


Figure 3.22 Total concentrations of phenolics in the two categories of *Solanum tuberosum* flowers.

3.5.1.4 Concentrations of individual phenolics in flowers

Phenolic acids

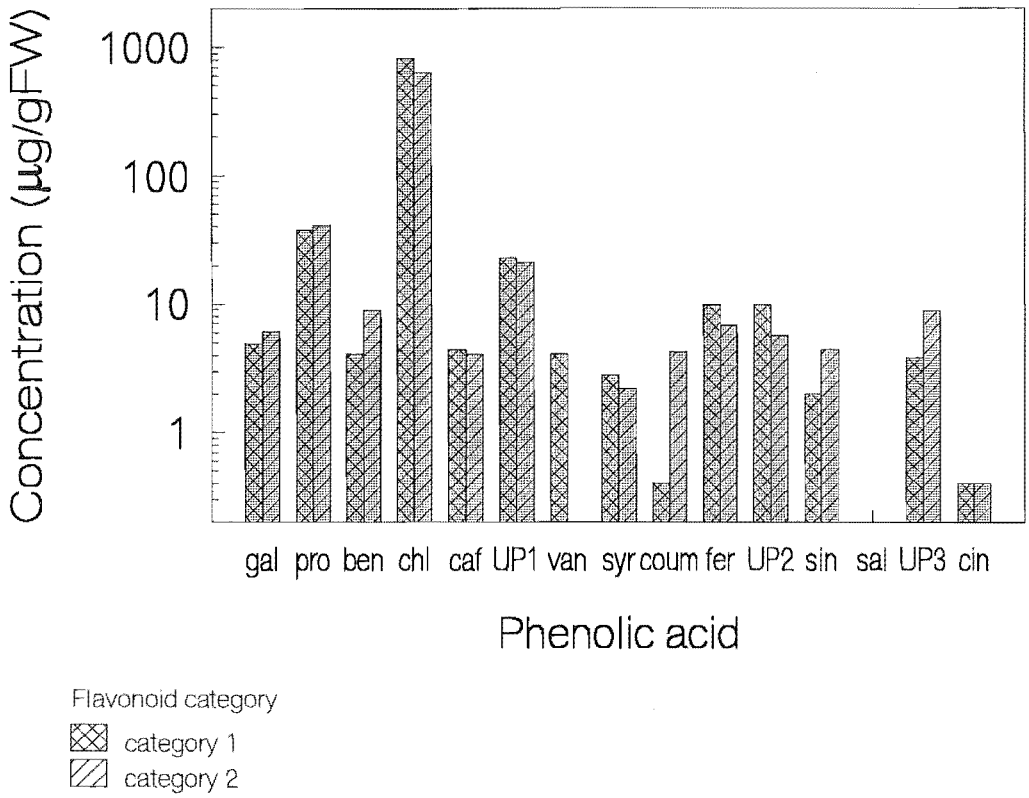
Chlorogenic acid was the major phenolic acid present in flowers, with moderate amounts of protocatechuic acid and UP1. Lower concentrations of gallic, *p*-hydroxybenzoic, caffeic, vanillic, syringic, *p*-coumaric, ferulic, UP2, sinapic and cinnamic acids were found, however salicylic acid was not detected in flowers. There was no significant difference in any of the individual phenolic acid concentrations between the two categories of flower, except for vanillic and *p*-coumaric acids (Figure 3.23). *p*-Coumaric acid concentrations were extremely low in category 1 flowers with higher concentrations in category 2 flowers, whilst vanillic acid was the reverse with no detectable vanillic acid in category 2 flowers, but significantly higher concentrations in category 1 flowers. The reasons for the differences in these phenolic acid concentrations related to flavonoid concentrations in category 1 and 2 flowers is not known.

Flavonoids

The major flavonoids found in flowers were the flavonols, Qu-gly 2a, Qu-gly 2b and rutin, with much lower concentrations of catechin, epicatechin, UF1, Qu-3-gal, Qu-3-glu, Km-3-rut and UF2 (0-50μg/gFW) and very low concentrations of eriodictyol and naringenin (0-5μg/gFW). A large difference between categories was shown by some of the individual flavonoids (Figure 3.24). As previously mentioned, category 1 flowers contained relatively high concentrations of Qu-gly 2a, Qu-gly 2b and rutin (average concentrations of 767, 376 and 663μg/gFW respectively), whilst category 2 flowers contained very low concentrations of Qu-gly 2a and Qu-gly 2b, and very high concentrations of rutin (average concentrations of 29, 31 and 1803μg/gFW respectively). A small, but significant, difference was observed for Km-3-rut with a higher concentration shown by category 2 flowers (Figure 3.24). There were no significant differences for any of the other flavonoids.

Anthocyanins

The major anthocyanins found in flowers were Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1) and Mv-3-(*p*-coumaroyl-rut)-5-glu (anth-2). Higher concentrations of Dp-3-rut, Mv-3-(*p*-coumaroyl-rut)-5-glu (anth-2) and a Pg-gly were found in category 2 flowers, but differences were not significant (Figure 3.25).



gal = gallic acid

pro = protocatechuic acid

ben = *p*-hydroxybenzoic acid

chl = chlorogenic acid

caf = caffeic acid

UP1 = unidentified phenolic acid 1

van = vanillic acid

syr = syringic acid

coum = *p*-coumaric acid

fer = ferulic acid

UP2 = unidentified phenolic acid 2

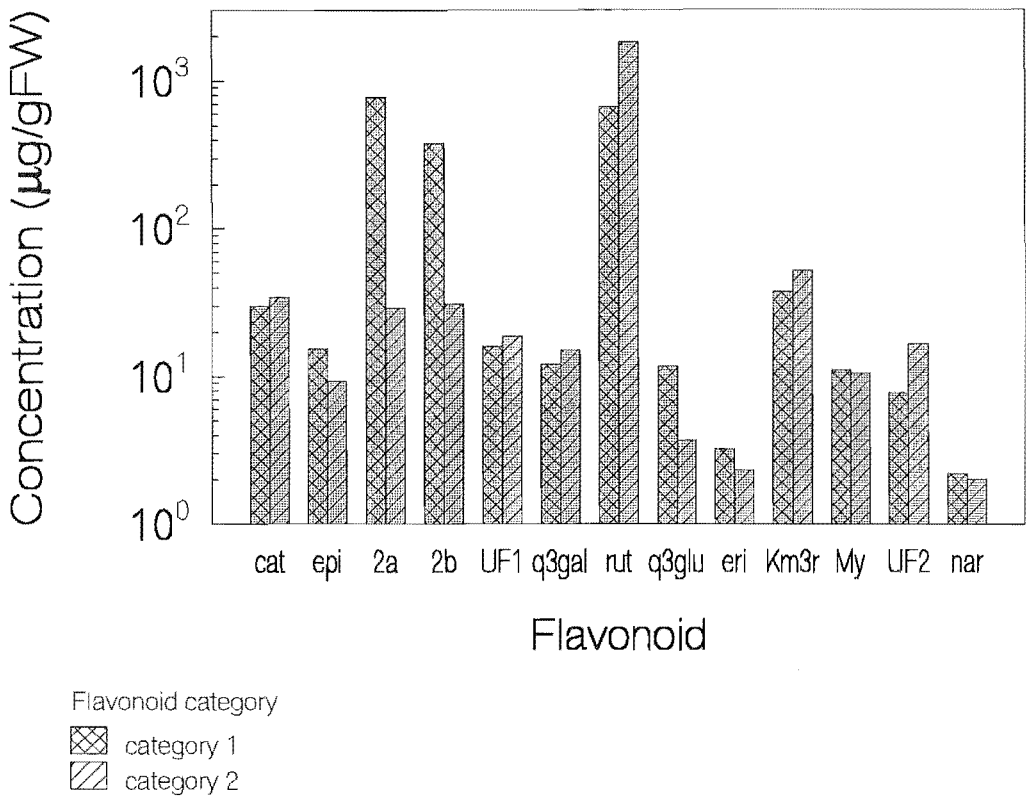
sin = sinapic acid

sal = salicylic acid

UP3 = unidentified phenolic acid 3

cin = cinnamic acid

Figure 3.23 Phenolic acid concentrations in the two categories of *Solanum tuberosum* flowers.



cat = catechin

epi = epicatechin

2a = quercetin-glycoside 2a (Qu-gly 2a)

2b = quercetin-glycoside 2b (Qu-gly 2b)

UF1 = unidentified flavonoid 1

q3gal = quercetin-3-galactoside (Qu-3-gal)

rut = rutin

q3glu = quercetin-3-glucoside (Qu-3-glu)

eri = eriodictyol

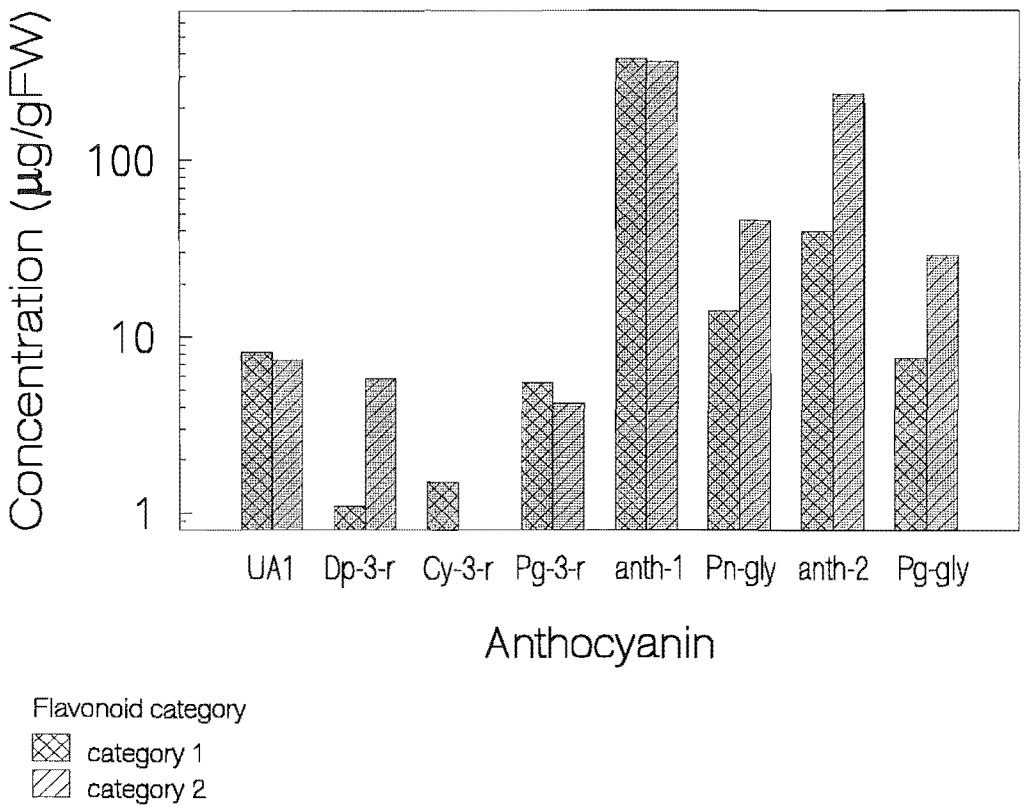
Km3r = kaempferol-3-rutinoside (Km-3-rut)

My = myricetin

UF2 = unidentified flavonoid 2

nar = naringenin

Figure 3.24 Flavonoid concentrations in the two categories of *Solanum tuberosum* flowers.



UA1 = unidentified anthocyanin 1

Dp-3-r = delphinidin-3-rutinoside

Cy-3-r = cyanidin-3-rutinoside

Pg-3-r = pelargonidin-3-rutinoside

anth-1 = petunidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside

Pn-gly = peonidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside

anth-2 = malvidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside

Pg-gly = pelargonidin-glycoside

Figure 3.25 Anthocyanin concentrations in the two categories of *Solanum tuberosum* flowers.

3.5.1.5 Total concentrations of phenolic acids, flavonoids and anthocyanins in leaves

Leaves contained similar concentrations of phenolic acids (between 200-1600µg/gFW, depending on the cultivar) and lower concentrations of flavonoids (between 500-2400 µg/gFW, depending on the cultivar) than flowers (Appendix 3). No anthocyanins were detected in the leaves of *S. tuberosum* cultivars.

The flavonoid pattern in leaves was similar to that observed in flowers, with the ratio of ([2a] + [2b]) / [rutin] also falling into two categories. Thus, cultivars with a high ratio in flowers also had a high ratio in leaves, and flowers with a very low ratio were also found to have a matching low value in leaves. Therefore, the following data are also presented in these two categories for leaves (Figures 3.26, 3.27, 3.28).

As previously found in flowers (Figure 3.22), there was no significant difference between the two categories of leaf for total phenolic acids, flavonoids or anthocyanins (Figure 3.26).

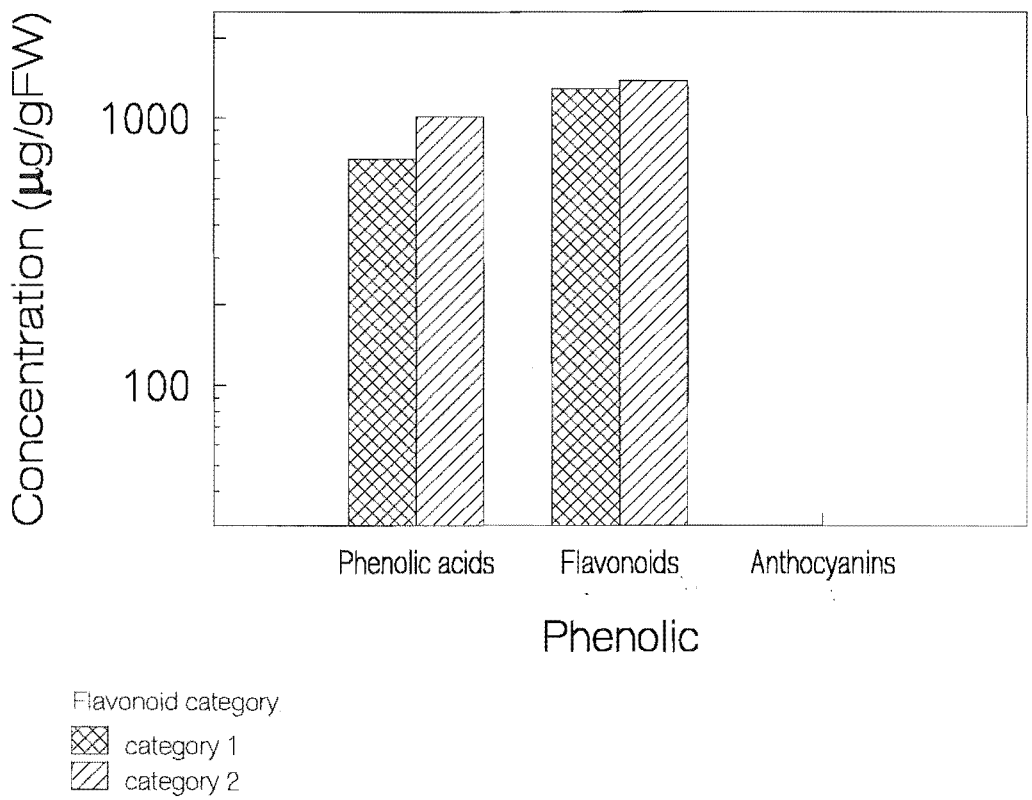


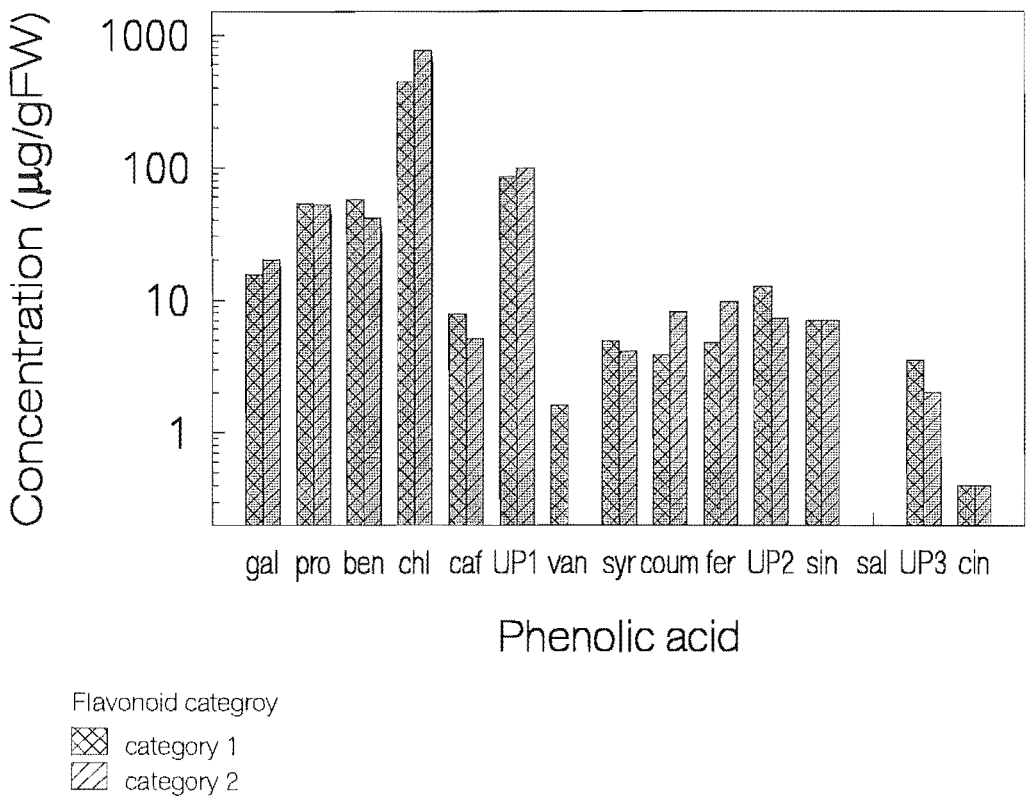
Figure 3.26 Total concentrations of phenolics in the two categories of *Solanum tuberosum* leaves.

3.5.1.6 Concentrations of individual phenolics in leaves

Phenolic acids

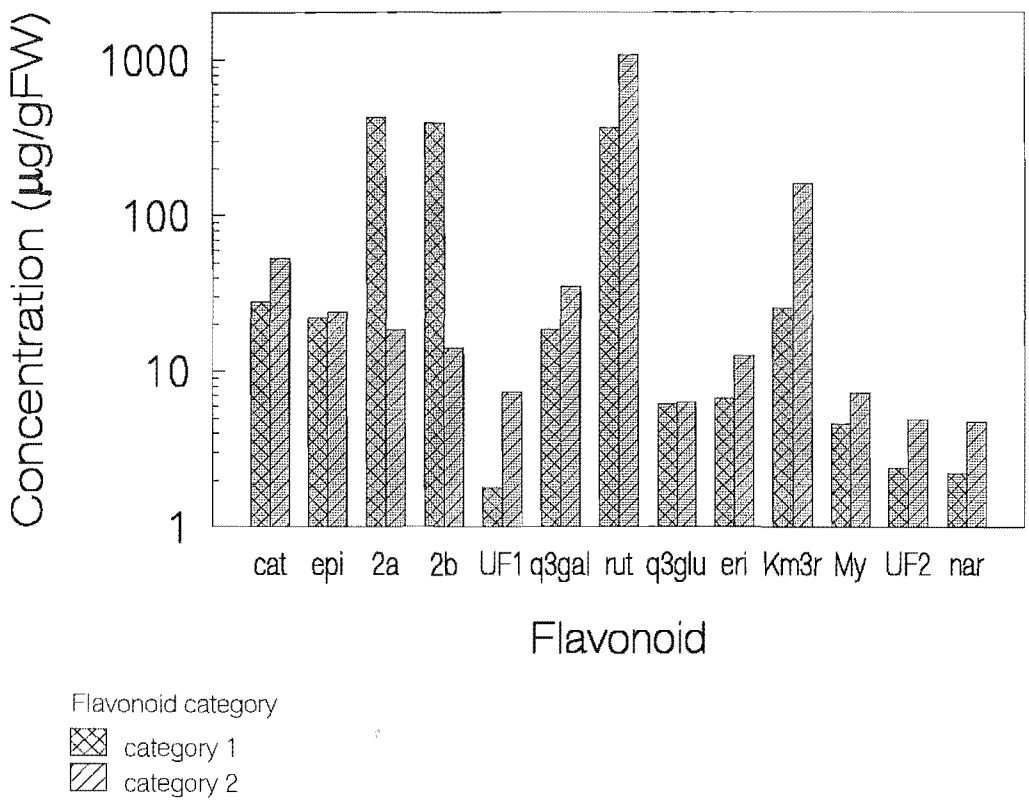
Individual leaf phenolic acids (Figure 3.27) showed a similar pattern to flowers (Figure 3.23), with the major phenolic acid being chlorogenic acid and with moderate amounts of protocatechuic acid, *p*-hydroxybenzoic acid and UP1. Low concentrations of UP2, UP3 and gallic, caffeic, vanillic, syringic, *p*-coumaric, ferulic, sinapic and cinnamic acids were

found but no salicylic acid was detected. There were no significant differences in leaf phenolic acids between categories 1 and 2 (Figure 3.27).



gal = gallic acid
pro = protocatechuic acid
ben = *p*-hydroxybenzoic acid
chl = chlorogenic acid
caf = caffeic acid
UP1 = unidentified phenolic acid 1
van = vanillic acid
syr = syringic acid
coum = *p*-coumaric acid
fer = ferulic acid
UP2 = unidentified phenolic acid 2
sin = sinapic acid
sal = salicylic acid
UP3 = unidentified phenolic acid 3
cin = cinnamic acid

Figure 3.27 Phenolic acid concentrations in the two categories of *Solanum tuberosum* leaves.



cat = catechin

epi = epicatechin

2a = quercetin-glycoside 2a (Qu-gly 2a)

2b = quercetin-glycoside 2b (Qu-gly 2b)

UF1 = unidentified flavonoid 1

q3gal = quercetin-3-galactoside (Qu-3-gal)

rut = rutin

q3glu = quercetin-3-glucoside (Qu-3-glu)

eri = eriodictyol

Km3r = kaempferol-3-rutinoside (Km-3-rut)

My = myricetin

UF2 = unidentified flavonoid 2

nar = naringenin

Figure 3.28 Flavonoid concentrations in the two categories of *Solanum tuberosum* leaves.

Flavonoids

A similar pattern of individual leaf flavonoids (Figure 3.28) was found as for flower flavonoids (Figure 3.24), with Qu-gly 2a, Qu-gly 2b and rutin having average concentrations of 428, 394 and 362μg/gFW respectively for category 1, and 19, 14 and 1071μg/gFW respectively for category 2. Leaf extracts also showed a clear difference in Km-3-rut between categories, with category 2 having a much higher concentration (Figure 3.28). As for flower extracts there was no significant difference in any of the other flavonoids.

3.5.2 Analysis of enzyme levels in different cultivars and correlation with anthocyanin, flavonoid and phenolic acid concentrations

The enzyme activities and phenolic concentrations were measured from field grown tubers of Arran Victory, Desirée, Red Flesh, Urenika and Whitu plants. The activity of phenylalanine ammonia-lyase (PAL) was not closely correlated with the final concentrations of anthocyanin, flavonoid or phenolic acids (Table 3.24). Cinnamic acid 4-hydroxylase (C4H), chalcone isomerase (CHI) and dihydroflavonol reductase (DHR) activities showed a good correlation between anthocyanin and phenolic acid concentrations in the skin, and CHI activity in the flesh was closely correlated to concentration of anthocyanins and flavonoids. Good correlations were also found between the activities of glycosyltransferase (GT) with some substrates and the phenolic concentrations, and between the activities of flavonoid 3'-hydroxylase (F3'H) with *p*-coumaric acid and naringenin as substrates and some phenolic concentrations (Table 3.24). Activities of flavanone 3-hydroxylase (F3H) showed no significant correlation with any of the three classes of phenolics (Table 3.24).

Table 3.24 A) Phenolic concentrations (μg/gFW) and B) enzyme activities (% of maximum activity for each enzyme) in different cultivars and correlations with phenolic concentrations.

A) Concentrations of phenolics

Phenolic		Whitu	Desirée	Arran Victory	Red Flesh	Urenika
Anthocyanin	skin	16.6	712.5	1700.2	2612.9	4376.4
	flesh	0	0	106.9	98.6	2320.3
Flavonoid	skin	149.7	336.9	289.6	335.3	146.4
	flesh	26.3	15.3	27.1	30.9	41.3
Phenolic acid	skin	1004.6	2292.3	2785.3	3239.1	3828.7
	flesh	188.5	337.5	638.6	736.1	1221.0

B) Enzyme activities and correlations

Enzyme assay *(substrate)*		Whitu	Desirée	Arran Victory	Red Flesh	Urenika	R^2 value (%) and significance		
PAL							Anth	Flav	Phen
	skin	40.35	100	90.38	55.37	59.12	25.4	50.7	72.1
	flesh	5.04	7.57	5.40	2.48	9.08	68.7	75.8	49.7
C4H									
	skin	51.34	43.42	51.05	50.31	100	97.3*	45.1	88.7
	flesh	1.22	13.30	3.06	2.49	9.12	31.2	97.5*	8.2
CHI									
	skin	2.38	1.03	1.70	16.01	33.23	96.0*	28.0	97.7*
	flesh	0.88	0.00	0.90	53.53	100	81.4	89.7	88.2
F3H									
(naringenin)	skin	60.78	44.69	48.72	68.75	23.87	51.8	17.0	31.5
	flesh	5.98	4.55	2.83	3.99	6.26	81.0	33.4	91.6
(eriodictyol)	skin	73.84	68.20	71.97	100	46.82	54.6	38.4	19.8
	flesh	10.71	7.99	3.35	5.53	10.34	86.6	25.4	93.1
DHR									
	skin	5.98	30.90	10.92	58.92	100	88.3	25.2	87.8
	flesh	9.52	32.1	14.31	17.47	22.43	13.0	85.4	2.5
F3'H									
(p-coumaric acid)	skin	75.00	42.55	47.51	41.03	68.21	80.5	97.0*	90.8
	flesh	15.94	72.51	20.61	46.79	62.93	23.6	77.7	15.7
(naringenin)	skin	12.80	50.58	94.65	89.06	100	94.8	14.1	92.4
	flesh	23.59	14.96	21.08	34.34	25.03	32.5	61.7	22.5
(kaempferol)	skin	1.53	59.5	27.26	58.54	30.86	41.7	83.3	64.4
	flesh	2.34	6.21	3.55	8.87	4.72	12.5	1.8	36.1
(apigenin)	skin	5.52	2.01	13.86	6.28	10.95	30.1	69.9	24.4
	flesh	2.94	3.90	3.22	2.05	2.42	40.9	77.1	35.4

continued...

B) Enzyme activities and correlations continued...

Enzyme assay *(substrate)*		Whitu	Desirée	Arran Victory	Red Flesh	Urenika	R^2 value (%) and significance		
GT							Anth	Flav	Phen
(quercetin/glucose)	skin	40.43	31.63	24.34	25.19	40.25	99.7**	91.8	76.4
	flesh	8.07	8.70	20.37	84.45	73.37	58.5	57.1	67.6
(luteolin/glucose)	skin	21.29	15.42	18.82	12.89	8.80	80.2	20.5	83.2
	flesh	0.90	11.22	3.57	4.39	5.85	8.5	83.6	1.1
(kaempferol/galactose)	skin	36.38	20.79	18.51	24.93	36.64	82	96.7*	98.2*
	flesh	0.47	8.53	7.26	30.73	28.81	55.5	50.3	71.2
(quercetin/galactose)	skin	63.47	42.45	31.18	34.04	27.87	89.3	14.5	96.9*
	flesh	9.06	1.65	4.33	35.25	20.50	29.5	42.6	32.5
(luteolin/galactose)	skin	17.94	21.29	20.79	18.22	9.26	98.2*	57.9	96.8*
	flesh	0	5.27	3.96	4.48	5.44	28.7	41.1	53.7
(kaempferol/xylose)	skin	ND	ND	ND	34.12	39.88	-	-	-
	flesh	ND	ND	ND	9.71	6.57	-	-	-
(quercetin/xylose)	skin	ND	ND	ND	20.89	33.45	-	-	-
	flesh	ND	ND	ND	9.16	11.80	-	-	-
(luteolin/xylose)	skin	ND	ND	ND	12.56	8.91	-	-	-
	flesh	ND	ND	ND	3.78	4.44	-	-	-

* significant at the 0.05 level

** significant at the 0.01 level

Anth - anthocyanin concentration (µg/gFW)

Flav - flavonoid concentration (µg/gFW)

Phen - phenolic acid concentration (µg/gFW)

PAL - phenylalanine ammonia-lyase

C4H - cinnamic acid 4-hydroxylase

CHI - chalcone isomerase

F3H - flavanone 3-hydroxylase

DHR - dihydroflavonol reductase

F3'H - flavonoid 3'-hydroxylase

GT - glycosyltransferase

ND not determined

- not enough data for regression analysis

3.5.3 Other *Solanum* species

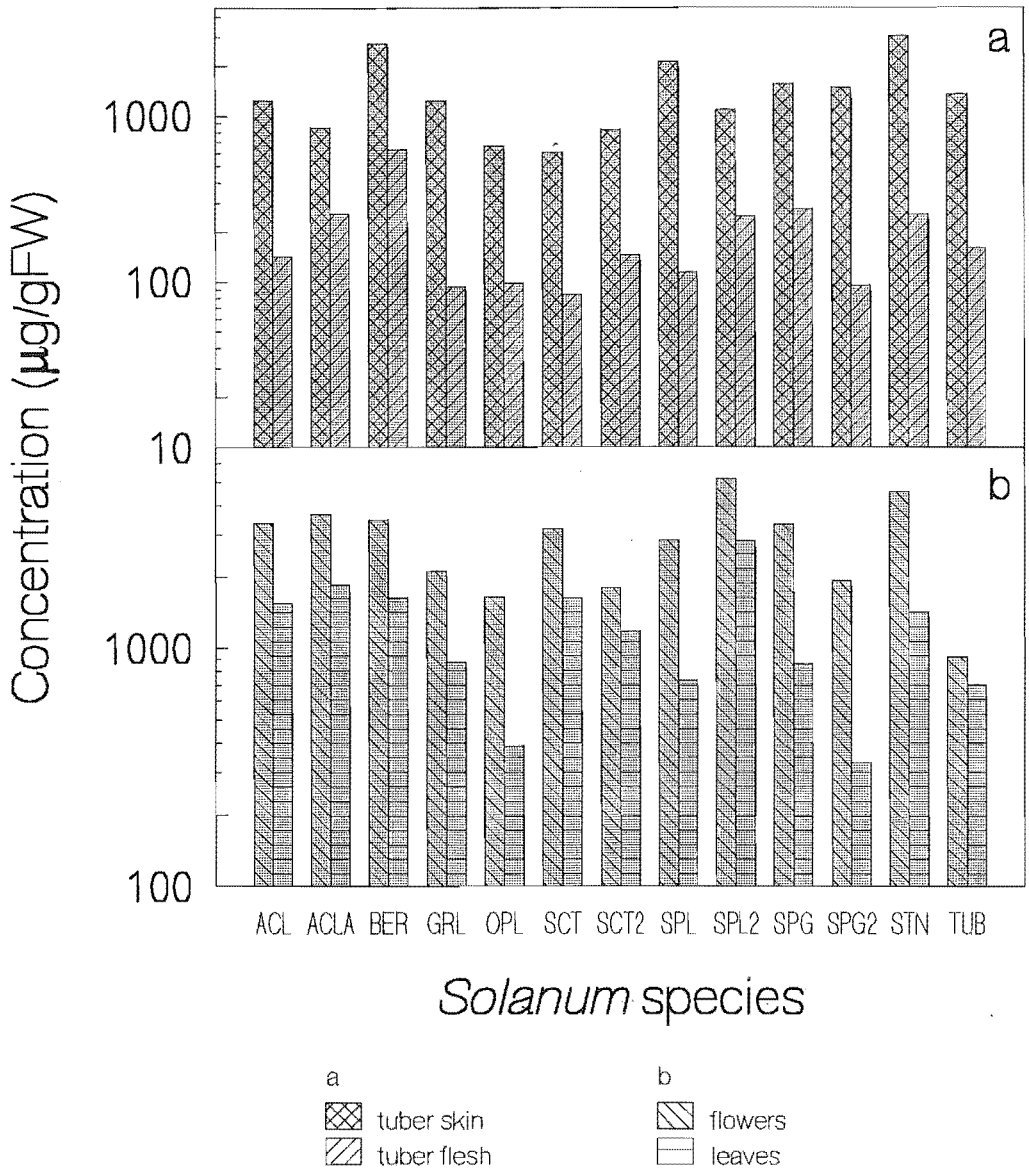
Characteristics of the other eight *Solanum* species used in these experiments are shown in Appendix 1 (Table A1.3). Phenolics were extracted from tubers (skin and flesh), flowers and leaves of these species and the visible λ_{max} recorded to indicate the identity of the major anthocyanidin present in the extracts (Table 3.25). The visible λ_{max} of all extracts (when present) was 538nm which indicated that the major aglycone was a glycoside of Mv, Dp or Pt.

Table 3.25 λ_{max} values of *Solanum* extracts.

Species	Species line	skin	flower	leaf
S. acaule subsp. *acaule*	ACL 2113	-	538	-
S. acaule subsp. *aemulans*	ACLAEM 3734	-	538	538
S. berthaultii	BER 4036	-	538	-
S. gourlayi	GRL 2480	538	538	538
S. oplocense	OPL 3777	-	538	-
S. sanctae-rosae	SCT 3269	-	538	538
S. sanctae-rosae	SCT 3779	538	538	538
S. sparsipilum	SPL 3488	538	538	538
S. sparsipilum	SPL 3563	538	538	538
S. speggazzinii	SPG 3745	538	538	-
S. speggazzinii	SPG 3791	538	538	-
S. stenotomum	STN 4711	-	538	-

- = no anthocyanin present

The extracts were analysed by analytical HPLC and the identities and concentrations of phenolic acids, flavonoids and anthocyanins determined. The identity of the major anthocyanin, usually Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1), was supported by the λ_{max} values. Concentrations of individual phenolic acids, flavonoids and anthocyanins for each species are shown in Appendix 4. Figures 3.29, 3.30 and 3.31 show the total concentration of compounds in these phenolic classes from the skin, flesh, flowers and leaves of these species. Average values of predominantly white skinned *S. tuberosum* cultivars are shown as a comparison, because the other *Solanum* species typically had mostly white skinned tubers. Concentrations of all phenolics were higher in the skin than in the flesh, and higher in flowers than in leaves for all species.



ACL = *S. acuale* subsp. *acaule* - ACL 2113

ACLA = *S. acaule* subsp. *aemulans* - ACLAEM 3734

BER = *S. berthaultii* - BER 4036

GRL = *S. gourlayi* subsp. *gourlayi* - GRL 2480

OPL = *S. oplocense* - OPL 3777

SCT = *S. sanctae-rosae* - SCT 3269

SCT2 = *S. santae-rosae* - SCT 3779

SPL = *S. sparsipilum* - SPL 3488

SPL2 = *S. sparsipilum* - SPL 3563

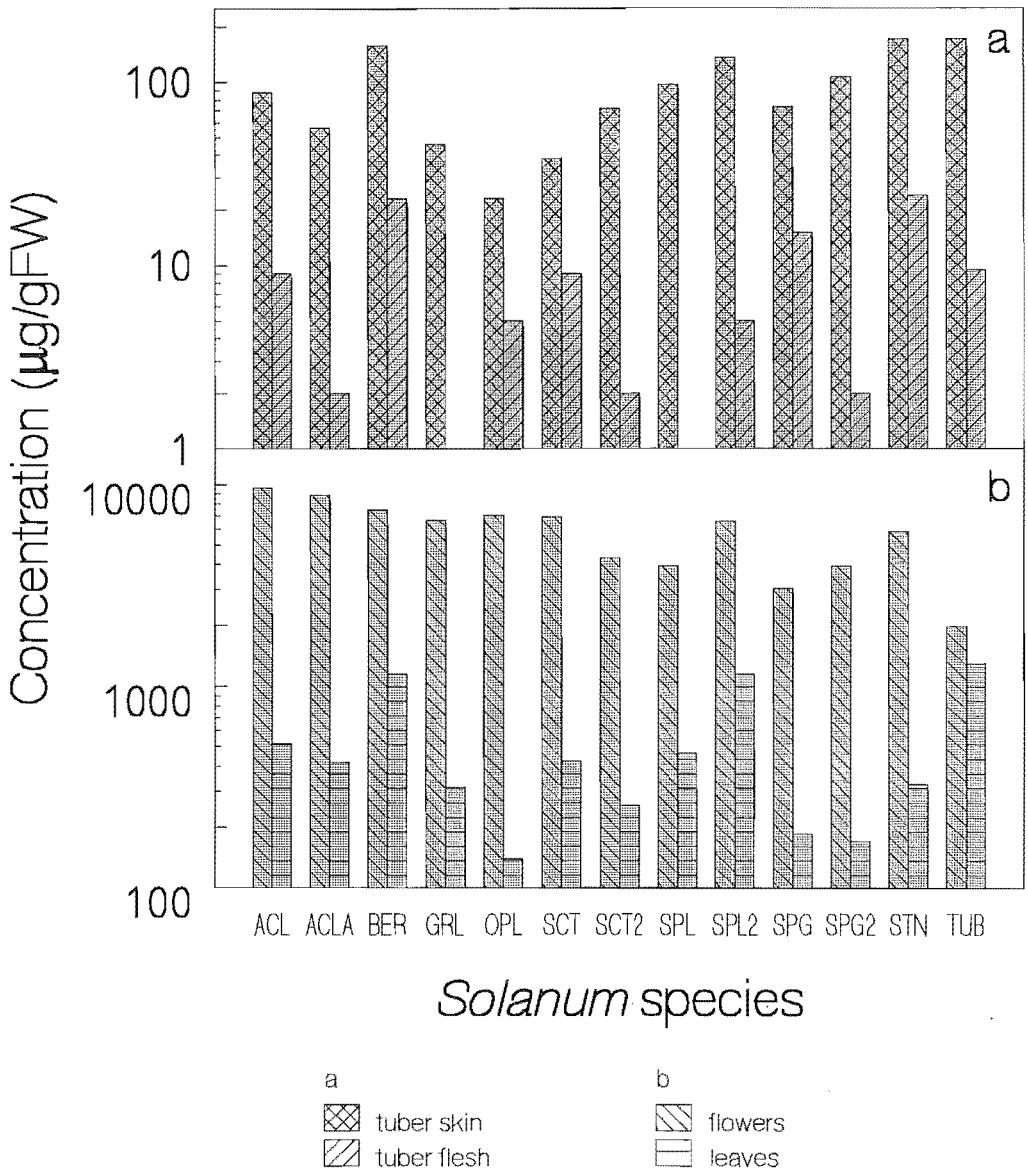
SPG = *S. speggazzinii* - SPG 3745

SPG2 = *S. speggazzinii* - SPG 3791

STN = *S. stenotomum* - STN 4711

TUB = *S. tuberosum*

Figure 3.29 Total phenolic acid concentration in a) skin and flesh, and b) flowers and leaves of *Solanum* species.



ACL = *S. acaule* subsp. *acaule* - ACL 2113

ACLA = *S. acaule* subsp. *aemulans* - ACLAEM 3734

BER = *S. berthaultii* - BER 4036

GRL = *S. gourlayi* subsp. *gourlayi* - GRL 2480

OPL = *S. oplocense* - OPL 3777

SCT = *S. sanctae-rosae* - SCT 3269

SCT2 = *S. santae-rosae* - SCT 3779

SPL = *S. sparsipilum* - SPL 3488

SPL2 = *S. sparsipilum* - SPL 3563

SPG = *S. speggazzinii* - SPG 3745

SPG2 = *S. speggazzinii* - SPG 3791

STN = *S. stenotomum* - STN 4711

TUB = *S. tuberosum*

Figure 3.30 Total flavonoid concentration in a) skin and flesh, and b) flowers and leaves of *Solanum* species.

3.5.3.1 Phenolic acids

Total phenolic acid concentration in these *Solanum* species varied from 600-2700 μg/gFW in the skin, 100-600μg/gFW in the flesh (Figure 3.29a), 1600-5100μg/gFW in flowers and 300-2800μg/gFW in leaves (Figure 3.29b), depending on the species. In skin, flower and leaf extracts the major phenolic acid was chlorogenic acid. In skin extracts chlorogenic acid accounted for 40-50% of the total phenolic acid content, with caffeic acid also present in high concentrations (10-30% of the total). In flowers and leaves 70-90% of the total phenolic acids was chlorogenic acid. Flesh phenolic acids comprised 30-40% protocatechuic acid, 20-30% chlorogenic acid and 20-30% *p*-coumaric acid (Appendix 4).

S. tuberosum flowers contained lower average concentrations of total phenolic acids when compared with the other eight *Solanum* species, whilst there was no difference in phenolic acid concentrations in the skin, flesh and leaves (Figure 3.29).

3.5.3.2 Flavonoids

Concentrations of total flavonoids ranged from between 20-170μg/gFW in skin, 0-20 μg/gFW in flesh (Figure 3.30a), 2000-9000μg/gFW in flower extracts and 150-3000 μg/gFW in leaf extracts (Figure 3.30b), depending on the species.

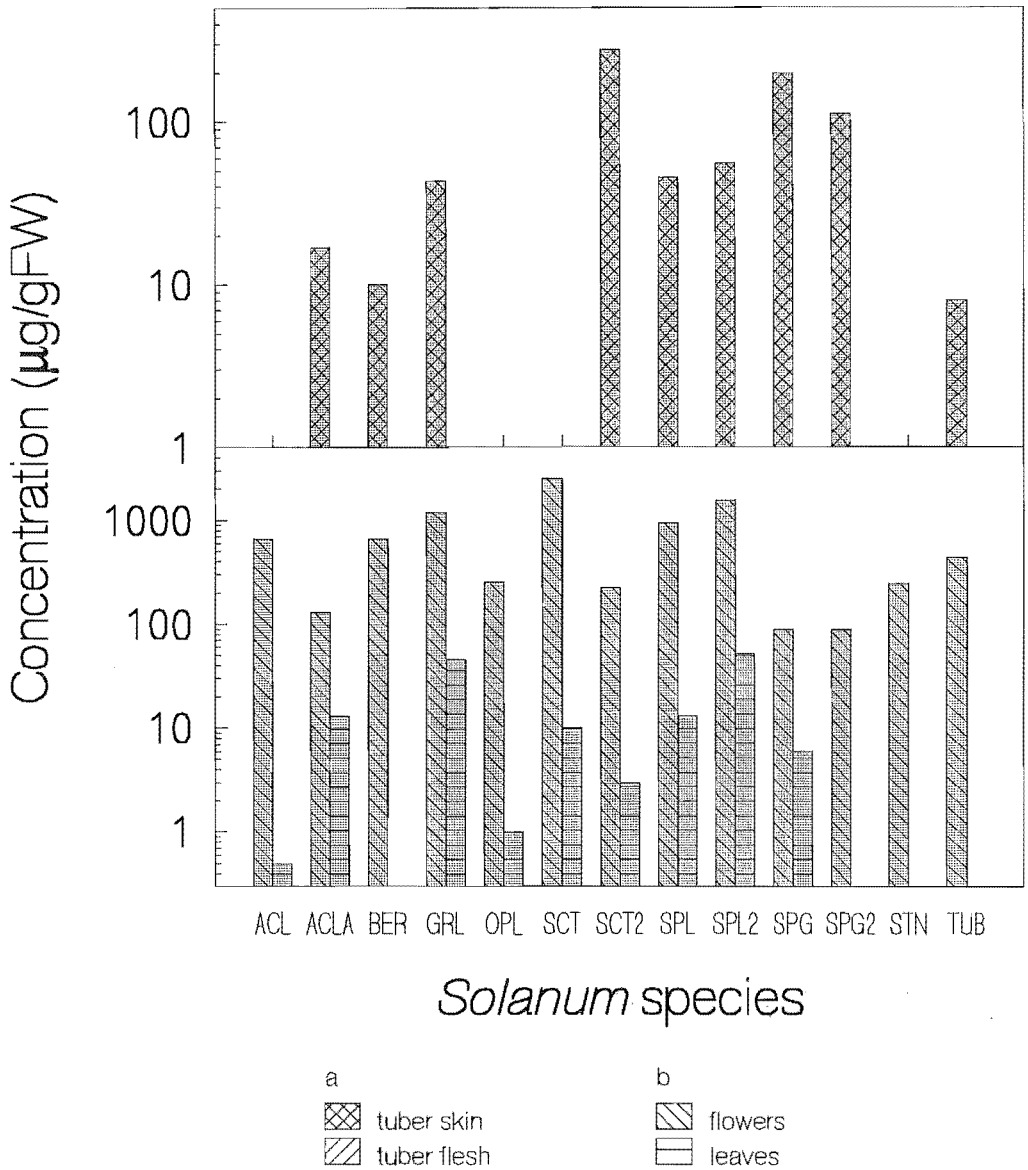
As previously found in *S. tuberosum* cultivars (Section 3.5.1.3), there appeared to be two different flavonoid patterns in the flowers and leaves, depending on the concentrations of 2a, 2b and rutin. All imported lines had a ratio of ([2a] + [2b]) / [rutin] under 0.06, except *S. sparsipilum* (SPL 3488) (but not SPL 3563), *S. speggazzinii* (SPG 3745 and SPG 3791) and *S. stenotomum* (STN 4711), which had much higher ratios (usually over one) (Table 3.26). The flowers and leaves of each line usually had a similar ratio.

In skin and flesh the major flavonoids were catechin, epicatechin, eriodictyol and naringenin, whereas the flavonoids in flowers and leaves were made up of Qu-glycosides 2a, 2b and/or rutin, with these accounting for 80-95% of the flavonoids in flowers and 60-80% in leaves (Appendix 4). *S. tuberosum* tubers and leaves contained high concentrations of flavonoids compared with other *Solanum* species, but flowers had lower concentrations.

Table 3.26 Concentrations of flavonoids 2a, 2b and rutin in *Solanum* species.

Species	*Solanum* species	extract	2a	2b	rutin	([2a] + [2b]) ÷ [rutin]
S. acaule	ACL 2113	flowers	12.9	35.0	8867.6	0.005
subsp. *acaule*		leaves	3.0	2.7	357.0	0.016
S. acaule	ACLAEM 3734	flowers	40.3	61.8	8204.5	0.012
subsp. *aemulans*		leaves	6.9	3.1	271.0	0.037
S. berthaultii	BER 4036	flowers	37.7	75.4	6536.9	0.017
		leaves	2.0	-	607.3	0.003
S. gourlayi	GRL 2480	flowers	-	16.8	5851.7	0.003
		leaves	-	7.6	214.8	0.035
S. oplocense	OPL 3777	flowers	35.0	18.2	5804.2	0.009
		leaves	-	4.9	80.4	0.061
S. sanctae-rosae	SCT 3269	flowers	2.8	14.3	6073.4	0.003
		leaves	-	1.0	276.8	0.004
S. sanctae-rosae	SCT 3779	flowers	11.2	16.3	3783.7	0.007
		leaves	3.9	1.4	111.8	0.047
S. sparsipilum	SPL 3488	flowers	1478.8	247.2	1768.9	0.976
		leaves	156.8	22.3	160.0	1.115
S. sparsipilum	SPL 3563	flowers	24.5	16.0	5460.8	0.007
		leaves	8.2	3.9	719.1	0.017
S. speggazzinii	SPG 3745	flowers	1602.5	116.3	1026.6	1.674
		leaves	6.1	26.8	13.6	2.419
S. speggazzinii	SPG 3791	flowers	2555.0	173.8	927.4	2.942
		leaves	20.7	6.4	10.7	2.533
S. stenotomum	STN 4711	flowers	1028.3	60.2	4309.2	0.253
		leaves	126.2	18.6	81.7	1.772

- = compound not detected



ACL = *S. acaule* subsp. *acaule* - ACL 2113

ACLA = *S. acaule* subsp. *aemulans* - ACLAEM 3734

BER = *S. berthaultii* - BER 4036

GRL = *S. gourlayi* subsp. *gourlayi* - GRL 2480

OPL = *S. oplocense* - OPL 3777

SCT = *S. sanctae-rosae* - SCT 3269

SCT2 = *S. santae-rosae* - SCT 3779

SPL = *S. sparsipilum* - SPL 3488

SPL2 = *S. sparsipilum* - SPL 3563

SPG = *S. speggazzinii* - SPG 3745

SPG2 = *S. speggazzinii* - SPG 3791

STN = *S. stenotomum* - STN 4711

TUB = *S. tuberosum*

Figure 3.31 Total anthocyanin concentration in a) skin and flesh, and b) flowers and leaves of *Solanum* species.

3.5.3.3 Anthocyanins

Variable concentrations of anthocyanins were found in the skins (between 0-300μg/gFW, depending on the species), whilst no anthocyanins were found in the flesh (Figure 3.31a). All tubers were predominantly white, although some showed purple colouration around the eyes. Flower anthocyanin concentrations ranged from 100-2500μg/gFW and leaves from 0-50μg/gFW, depending on the species (Figure 3.31b). The major anthocyanin was always Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1) with much lower concentrations of Mv-3-rut and Mv-3-(*p*-coumaroyl-rut)-5-glu (anth-2). Low concentrations of UA1 and Cy-3-rut were also occasionally found in the flowers (Appendix 4, Table A4.3).

3.5.4 Effect of disease

Two plants of *S. sanctae-rosae* (SCT 3778) were found to be diseased, probably infected with *Phytophthora infestans* (late blight). These plants (plant numbers 4 and 8) had diseased tubers, so these tubers were analysed separately from the others in this line (plant numbers 6, 12 and 13). Both the total phenolic acids and flavonoids showed greatly increased concentrations in the skin and flesh of diseased tubers, whilst the anthocyanin content was higher in the skin of healthy tubers than diseased tubers (Figure 3.32). No anthocyanins were found in the flesh of healthy or diseased tubers.

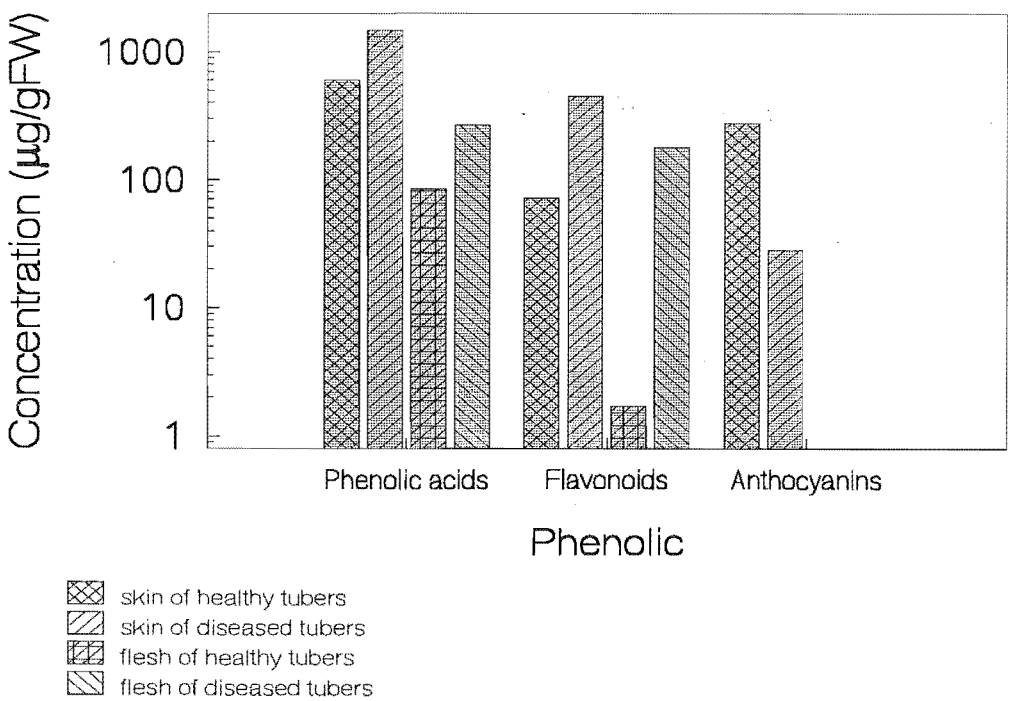
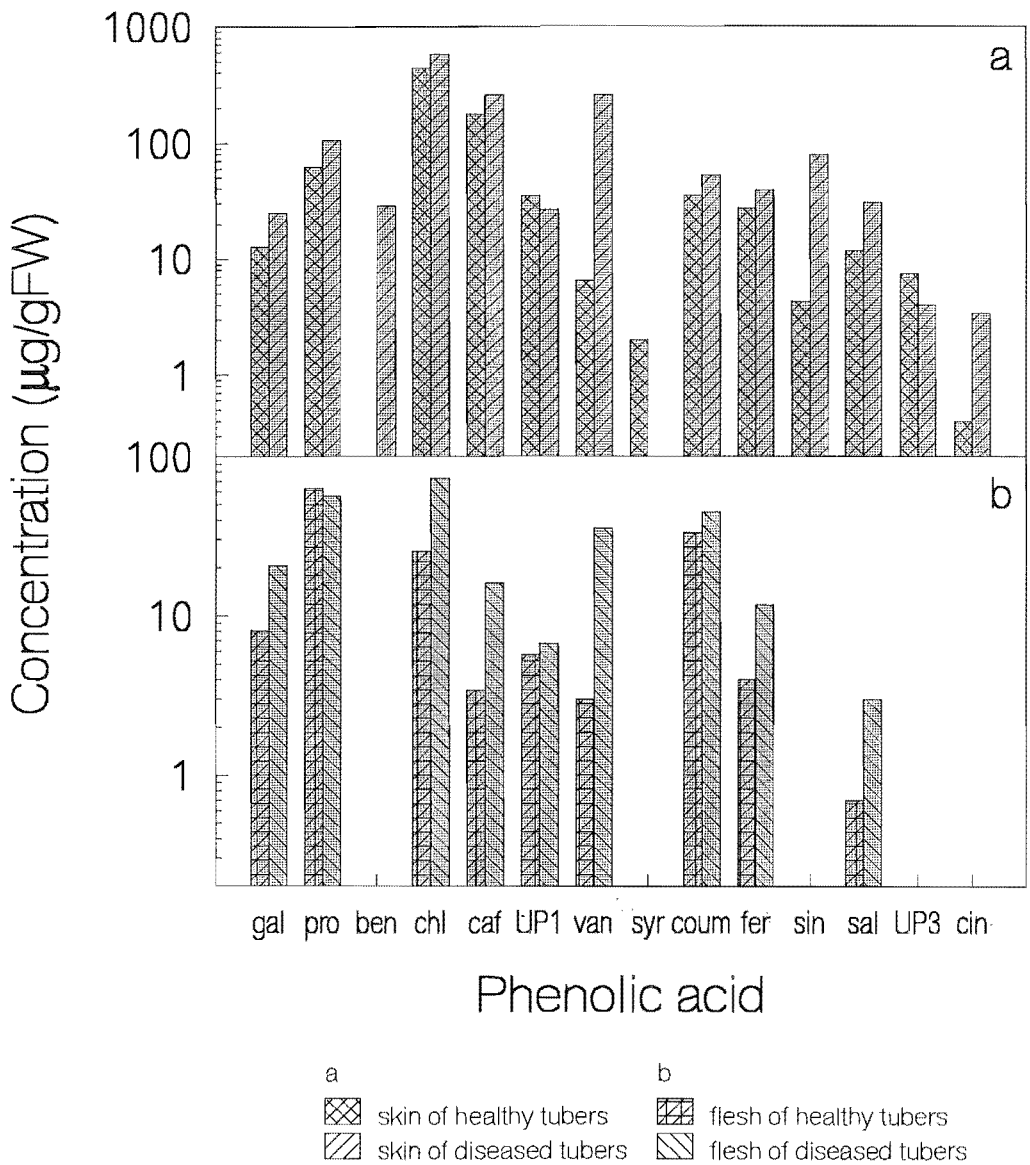


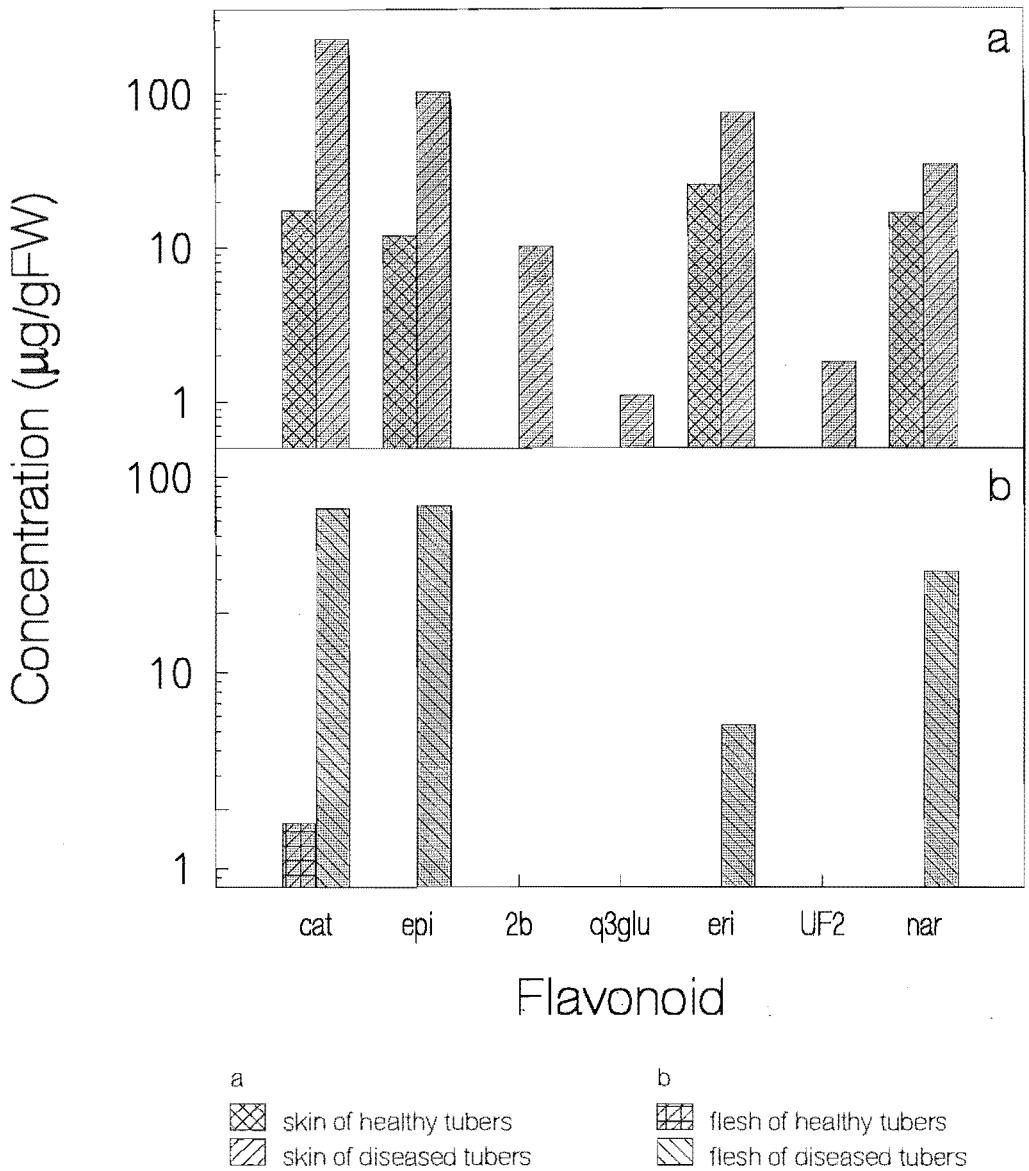
Figure 3.32 Total concentrations of phenolic acids, flavonoids and anthocyanins in healthy and diseased *Solanum sanctae-rosae* tubers.

In the diseased tubers, significant increases were seen for gallic acid, *p*-hydroxybenzoic acid, chlorogenic acid, caffeic acid, vanillic acid, ferulic acid, sinapic acid and salicylic acid, but not for syringic acid, UP1 and UP3, and protocatechuic acid (Figure 3.33).



gal = gallic acid
pro = protocatechuic acid
ben = *p*-hydroxybenzoic acid
chl = chlorogenic acid
caf = caffeic acid
UP1 = unidentified phenolic acid 1
van = vanillic acid
syr = syringic acid
coum = *p*-coumaric acid
fer = ferulic acid
sin = sinapic acid
sal = salicylic acid
UP3 = unidentified phenolic acid 3
cin = cinnamic acid

Figure 3.33 Phenolic acid concentrations in a) skin and b) flesh of healthy and diseased *Solanum sanctae-rosae* tubers.



cat = catechin

epi = epicatechin

2b = quercetin-glycoside 2b (Qu-gly 2b)

q3glu = quercetin-3-glucoside (Qu-3-glu)

eri = eriodictyol

UF2 = unidentified flavonoid 2

nar = naringenin

Figure 3.34 Flavonoid concentrations in a) skin and b) flesh of healthy and diseased *Solanum sanctae-rosae* tubers.

Flavonoid concentrations also showed significant increases in diseased tubers. The healthy tubers contained catechin, epicatechin, eriodictyol and naringenin in the skin, but only catechin in the flesh, whereas diseased tubers contained these flavonoids in much greater concentrations, and additionally contained low concentrations of Qu-gly 2b, Qu-3-glu and UF2 in the skin, and epicatechin, naringenin and eriodictyol in the flesh (Figure 3.34).

No anthocyanins were found in the flesh, but healthy tubers contained Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1) and Mv-3-rut in the skin, whereas diseased tubers contained lower concentrations of Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1) and no Mv-3-rut in the skin (not shown), however the anthocyanin concentration of these *S. sanctae-rosae* tubers was too variable to show a significant effect.

3.6 Discussion

3.6.1 Separation and identification of the phenolics

TLC (one-dimensional) was useful for quick screening and identification of anthocyanins and also for the identification of anthocyanin aglycones after acid hydrolysis. Phenolic acids and flavonoids could not be separated by one dimensional TLC, but were separable by 2-D PC although Rf values tended to be variable and resolution poor, or by 2-D TLC which, although it gave improved resolution and reliability over PC, was too expensive for routine analysis of numerous samples, and was not quantitative. Even so, 2-D TLC was still useful for the identification of compounds by calculation of the Rf values and the colours of compounds with the use of spray reagents, such as NA-reagent, and was used to complement the data obtained by HPLC in the identification of the phenolics. The HPLC separation of the potato phenolics showed considerable advantages over separation by TLC, with improved detection and greater resolution of the compounds, and was used for quantitative analyses. The greater resolution of compounds by HPLC was shown particularly for the anthocyanins where, using TLC only the major anthocyanins could be detected, whereas the use of HPLC often revealed another three to four minor anthocyanins.

The HPLC system with diode-array detector allowed the spectrum of each individual eluting compound to be recorded, which assisted greatly in identification of phenolics. For the routine analysis of extracts, the use of a variable wavelength detector enabled all compounds to be quantified in a single run. HPLC retention times varied slightly over a period of time due to ageing of the column and differences in solvents, however

chlorogenic acid and the major anthocyanins were always readily identifiable and these were used to adjust the retention times of other compounds for comparison against standards. The elution gradient proved adequate for the separation of most of the phenolic acids and flavonoids, however improved separation would have been useful for the anthocyanins, because Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1) and Pn-3-(*p*-coumaroyl-rut)-5-glu had similar retention times, as did Mv-3-(*p*-coumaroyl-rut)-5-glu (anth-2) and Pg-3-(*p*-coumaroyl-rut)-5-glu (anth-3). However, the similar retention times of these compounds was not a problem in this study because it was found that neither Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1) and Pn-3-(*p*-coumaroyl-rut)-5-glu, nor Mv-3-(*p*-coumaroyl-rut)-5-glu (anth-2) and Pg-3-(*p*-coumaroyl-rut)-5-glu (anth-3) occurred together in any one sample (confirmed by TLC of the aglycones). My and UF2 also had similar retention times to these anthocyanins. Improved separation would have been useful because My and UF2 could not be detected when these anthocyanins were present.

The identities of the anthocyanins were determined by TLC, HPLC and MS, using unhydrolysed glycosides and/or the hydrolysed aglycones. It was found that purple coloured tubers contained Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1) and Mv-3-(*p*-coumaroyl-rut)-5-glu (anth-2), red coloured tubers contained Pn-3-(*p*-coumaroyl-rut)-5-glu and Pg-3-(*p*-coumaroyl-rut)-5-glu (anth-3), and flowers contained Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1) and Mv-3-(*p*-coumaroyl-rut)-5-glu (anth-2). The current HPLC method would be applicable to many other plant species to quantify phenolic acids, flavonoids and/or anthocyanins, although slight changes may be needed to the elution gradient depending on the compounds present, to ensure an appropriate separation of compounds. A modified form of this method has been used previously to separate apple flavonoids (Lister, 1994).

3.6.1.1 Flavonoids

In this study, only the flavanone, flavonol and flavan-3-ol (and anthocyanin) classes of flavonoid were found in potato plants (tubers, flowers or leaves). Other studies (Harborne, 1960a; 1962; 1967; Wietschel and Reznik, 1980a and b) have also found these classes, plus compounds in the flavone class of flavonoids. Qu-3-glu, rutin and Km-3-rut have previously been found in *S. tuberosum* petals, leaves and seeds (Harborne, 1967), and were also found in this study. Harborne (1962) found four glycosidic types present in flavonoids from tuber-bearing *Solanum* species; 3-glu, 3-rut, 3-sophoroside, and 3-(2^G-glu-rut). Qu-3-(2^G-glu-rut) was found to occur in the flowers of some cultivars (e.g. Arran Victory), but not in others (Harborne, 1962). In this study only the 3-glu and 3-rut glycosides were identified. However, it is likely that either Qu-

gly 2a or Qu-gly 2b is Qu-3-(2^G-glu-rut) because all the available evidence supports this hypothesis. Evidence includes data from TLC, acid hydrolysis, and spectral analysis, including the use of shift reagents, as well as the distribution pattern of this compound mainly in the flowers and leaves, and the fact that these compounds (Qu-gly 2a and 2b) occurred in only some species and cultivars (e.g. Arran Victory). The identity of the other compound (Qu-gly 2a or 2b) is unknown, except that it also is a Qu-glycoside containing glucose and rhamnose, but it is likely that it is the Qu-3-(2^G-glu-rut), but with the addition of an acyl group, probably *p*-coumaric acid. There remain two unidentified flavonoids, UF1 and UF2, as well as a number of lesser peaks that were present in some cultivars and were not identified. It is suggested that UF1 and UF2 were flavonols (probably Qu and/or Km), and it is possible that they were glycosylated with the 3-sophoroside, because these compounds have been found previously in potatoes (Harborne, 1962; Wietschel and Reznik, 1980a and b).

Km-3-(2^G-glu-rut) has been found in *S. tuberosum* petals (Harborne, 1962), and Km-3-sophoroside-7-rha and Km-3-sophorotrioside-7-rha have been found by other researchers, but are restricted to the seeds (reviewed in Harborne, 1967). However, seeds were not considered, nor were these three flavonoids identified in this study. Of the free flavonol aglycones, My was found in most cultivars by HPLC analysis and Km was detected in small quantities in flowers using HPLC, however Km, Qu and My aglycones were identified in some tuber and flower extracts by 2-D TLC.

3.6.2 *Solanum tuberosum* cultivars

In general, tuber skin and flesh, and flower and leaf extracts from *S. tuberosum* cultivars all showed different and characteristic HPLC chromatograms at the four wavelengths studied (280, 313, 350 and 530nm), with the chromatograms for tubers being significantly different from those of flowers and leaves.

3.6.2.1 Tubers

Anthocyanins

The three major anthocyanins occurring in purple tubers were identified as Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1), Mv-3-(*p*-coumaroyl-rut)-5-glu (anth-2) and Mv-3-rut. The medium purple coloured tubers (such as Arran Victory and Blue Derwent) usually contained high concentrations of Pt-3-(*p*-coumaroyl-rut)-5-glu and low concentrations of Mv-3-(*p*-coumaroyl-rut)-5-glu and Mv-3-rut, whereas dark purple/black tubers (such as Urenika and Stage II Blue) contained similar concentrations of Pt-3-(*p*-coumaroyl-rut)-5-glu and Mv-3-rut as medium purple tubers, but additionally contained extremely high

concentrations of Mv-3-(*p*-coumaroyl-rut)-5-glu. Identification of the predominant anthocyanin (anth-2) in Urenika as Mv-3-(*p*-coumaroyl-rut)-5-glu agrees with the findings of previous researchers (Harborne, 1960a; Simmonds and Harborne, 1965) (since Urenika and Congo appear to be the same, or at least very similar cultivars).

Pg-3-(*p*-coumaroyl-rut)-5-glu (anth-3), Pg-3-rut and Pn-3-(*p*-coumaroyl-rut)-5-glu were the three major anthocyanins occurring in red skinned tubers, with Pg-3-(*p*-coumaroyl-rut)-5-glu always present in the highest concentration. Differences between pink (e.g. Desirée) and darker red (e.g. Red Flesh) tubers appeared to be caused by increased concentrations of all these anthocyanins in the darker red tubers, and also the presence of UA4 which was found in darker red tubers only (Appendix 3, Table A3.3). The identification of anthocyanins in red tubers agrees with the findings of Sachse (1973) who found Pg-3-(*p*-coumaroyl-rut)-5-glu and Pn-3-(*p*-feruloyl-rut)-5-glu in Desirée tubers with the latter anthocyanin more recently being identified as having a *p*-coumaroyl instead of a feruloyl acyl group (reviewed in Mazza and Miniati, 1993).

Therefore, it appears that the major anthocyanins in purple tubers were the methylated derivatives of a trihydroxylated B-ring (Pt and Mv), whilst in the red tubers the mono-hydroxylated B-ring (Pg) and the methylated derivative of the dihydroxylated B-ring (Pn) were found. This indicates that the "anthocyanin 5'-hydroxylase" enzyme activity is not present in the red tubers, but is present in the purple tubers. The "anthocyanin methyltransferase" enzyme appeared to be very active in purple tubers because only low concentrations of Dp-glycosides were found, most of these having been methylated to form Pt or Mv, which occurred at high concentrations in purple tubers.

Because the minor anthocyanins were present at such low levels in the plant it was difficult to purify and identify any but the major anthocyanins (anth-1, anth-2 and anth-3), so the identifications of other more minor anthocyanins were based on λ_{max} values and co-elution with standards. However, the limited number of standards and the possibility of the co-elution of two anthocyanins together make these identifications of minor anthocyanins tentative, at best. It was deemed unnecessary for this work to identify absolutely each anthocyanin, since emphasis is focused on the biochemistry of the anthocyanins as a class of compounds, rather than the individual anthocyanin molecules.

In this study, all of the six common anthocyanidins were found over the range of cultivars and tissue types studied, which is in agreement with the results found by Harborne (1967). Harborne (1960a; 1964; 1967) found that anthocyanins from *Solanum* species are glycosylated with glucose and rhamnose only, acylated only with *p*-coumaric

acid, and contain only the 3-(*p*-coumaroyl-rut)-5-glu and 3-rut derivatives of the common anthocyanin aglycones. However, in this study, a number of other minor anthocyanins appearing on the HPLC chromatograms suggested that *S. tuberosum* contains more than only these two glycosidic patterns. For example, Red Flesh skin extracts were found to contain up to four Pg-glycosides. Also in contrast to Harborne (1960a; 1964; 1967), Bobbio *et al.* (1987) reported Mv-3-sophoroside-5-glu and Mv-3-(di-caffeoyl-sophoroside)-5-glu in berries of *S. americanum*. It is likely that the early work of Harborne (1960a; 1964; 1967) using PC or TLC methods did not detect these minor anthocyanins. Other plants have different patterns of glycosylation with a number of different sugars. For example, apple skins contain the Cy-glycosides of 3-ara, 3-ara-gal, 7-ara, 3-gal, 3-gentiobioside, 3-glu and 3-xyl (Lister, 1994). Similarly, blueberries have been found to contain a number of different glycosylation patterns and also different aglycones occurring in the same cultivar. For example, Gao and Mazza (1994) found that a number of blueberry cultivars each contained twenty five different anthocyanins which were the acylated and non-acylated forms of the 3-ara, 3-gal, and 3-glu of Dp, Cy, Pt, Pn and Mv.

Flavonoids

The major flavonoids in the tubers were identified as the flavanones, naringenin and eriodictyol, the flavan-3-ols, catechin and epicatechin, and the flavonol, Km-3-rut, with the flavonol Qu-glycosides, 2a and 2b, also being found in some tubers at lower concentrations. Epicatechin was not found in the flesh of tubers, although it was present in the skin, flowers and leaves.

Tubers contained very low concentrations of flavonoids, and there was only a slightly higher flavonoid concentration in tubers containing anthocyanins. This suggests that in different cultivars the biosynthetic pathway is either very active with compounds going down the anthocyanin branch only and not into other flavonoid pathways, as in coloured tubers, or the whole flavonoid pathway (including the anthocyanin pathway) may have only low activity, as in white tubers. In addition, the relatively high concentrations of naringenin and eriodictyol precursors found in tubers supports the suggestion that the precursors coming into the general flavonoid pathway do not follow any branch except the anthocyanin route, and the absence or low concentrations of other flavonoids is not because of a lack of precursors. It suggests that there is a "block" in the flavonoid pathways (e.g. at the flavonol synthase enzyme in the flavonol pathway), but not in the anthocyanin pathway.

Red skinned tubers contained slightly higher concentrations of Km-3-rut than purple or white skinned tubers. This may be related to the fact that only red skinned tubers

contained Pg-glycosides; both Km and Pg having only the 4'-hydroxyl group on the B-ring, and suggests that flavonoid and anthocyanin classes maybe synthesised in parallel pathways.

Phenolic acids

Tuber skins contained high concentrations of phenolic acids, with much lower concentrations of phenolic acids being found in the tuber flesh. Reeve *et al.* (1969a) also found that chlorogenic acid was more concentrated in the outer tissues of tubers. The identities of the phenolic acids in the skin and flesh were similar, however the relative amounts of individual phenolic acids varied considerably between cultivars. The major phenolic acid in both the skin and flesh of tubers was chlorogenic acid. In the skin, moderate amounts of protocatechuic acid, sinapic acid, vanillic acid and UP1 were found, together with low concentrations of the other phenolic acids whilst, in the flesh the highest concentrations were found of chlorogenic acid and protocatechuic acid, with lower concentrations of *p*-coumaric acid, vanillic acid and UP1, with only very low concentrations of the other phenolic acids. Therefore, a large proportion of the precursors continue along the phenolic acid pathways, mostly to form chlorogenic acid, rather than going along the flavonoid and anthocyanin pathways, especially in white tubers.

There was a good correlation between anthocyanin and phenolic acid (in particular chlorogenic acid) concentrations. White tubers (containing little or no anthocyanin) contained low concentrations of most phenolic acids, whilst coloured tubers (containing high concentrations of anthocyanin) contained high concentrations of most phenolic acids, with coloured tubers generally containing twice the concentration of phenolic acids of white tubers. This suggests that the production of anthocyanins is not simply a matter of diverting phenolics from one pathway to another, but that the level of the whole phenylpropanoid biosynthesis appears to be increased, with increased production of almost all of the extracted phenolics, together with a huge increase in anthocyanin production (from 0μg/gFW in the white tubers up to 7000μg/gFW in the purple/black tubers). Therefore, in tubers the control of anthocyanin biosynthesis appears to be by substrate limitation, rather than regulation of the different pathways with the same amount of substrate being produced but diverted to other pathways. As mentioned previously, the flavonoids only showed a slight increase in coloured tubers compared with white tubers, indicating that only the flow of metabolites though the phenylpropanoid pathway and anthocyanin branch of flavonoid biosynthesis was increased, whilst the flow through other flavonoid pathways remained similar.

Using HPLC, de Sotillo *et al.* (1994a and b) found chlorogenic acid, gallic acid, protocatechuic acid and caffeic acid to be the major phenolic acids present in potato skins. Lyon and Barker (1984), Malmberg and Theander (1985), Kumar *et al.* (1991) and Ramamurthy *et al.* (1992) also reported some of the same phenolic acids as de Sotillo (1994a and b), and also reported chlorogenic acid as the major constituent in the peels and/or flesh of potato tubers. In this present study, the same major phenolic acids were detected as those found by these researchers, however in this study a number of additional phenolic acids were detected and identified. Previous published results which compare more closely to those in this present investigation were found by Cvikrová *et al.* (1994) in an analysis of bud meristematic tissue in sprouting tubers. They found caffeic, chlorogenic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, salicylic and vanillic acids, with chlorogenic acid accounting for 60-90% of the total phenolic acid concentration. Other similar results include those found by Sosulski *et al.* (1982) and Onyeneho and Hettiarachchy (1993) (Table 3.2), although the procedures used in this investigation for the detection of minor phenolic acids appeared to be better than those in any of these other studies.

3.6.2.2 Flowers

Anthocyanins

The predominant anthocyanin in flowers from cultivars with purple tubers was Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1), with lower concentrations of Mv-3-(*p*-coumaroyl-rut)-5-glu (anth-2) whereas, in flowers from red skinned cultivars the predominant anthocyanin was Pn-3-(*p*-coumaroyl-rut)-5-glu. Pg-3-(*p*-coumaroyl-rut)-5-glu (anth-3) was not found in the flowers from any cultivar, even in the light pink flowers from Red Flesh where it was the major anthocyanin in the tubers of this cultivar. None of the flowers from red skinned cultivars with red tubers were a dark purple - all were white, pink or light purple, whereas flowers from cultivars with purple tubers ranged from white to dark purple. So, for each cultivar, the flowers contained similar types of anthocyanin as the tubers (with the notable exception of the absence of Pg-3-(*p*-coumaroyl-rut)-5-glu in the flowers), and therefore the pigments present in the tubers and flowers of these cultivars appeared to support the proposed general scheme for the genetic control suggested for *S. tuberosum* (Section 1.7.2). The other exception was that some flowers contained low concentrations of Cy-3-rut, whilst no Cy-glycosides were found in tubers.

Flavonoids

The major flavonoids found in flowers were rutin and the Qu-glycosides, 2a and 2b; other minor flavonoids detected included catechin, epicatechin, Km-3-rut, My, Qu-3-gal, Qu-3-glu, UF1 and UF2. The flavonoid pattern in flowers fell into two categories;

flowers with high concentrations of flavonols, Qu-glycosides 2a and 2b, and rutin, and flowers with similar, or higher, concentrations of rutin, but very low concentrations of Qu-glycosides 2a and 2b, so that the ratio of ([2a] + [2b]) / [rutin] was indicative of the category. Most of the *S. tuberosum* cultivars had high concentrations of Qu-glycosides 2a and/or 2b, with only six cultivars having low concentrations. This did not appear to be related to the anthocyanin concentration in flowers, but it was interesting to note that all these cultivars had white tubers. In contrast to tubers, flowers generally contained high concentrations of flavonoids, but low concentrations of the flavonoid intermediates naringenin and eriodictyol. Therefore, the regulation of flavonoid biosynthesis in flowers appears to be different from that in tubers, with flowers containing high concentrations of flavonoids as well as anthocyanins.

Phenolic acids

Flowers contained lower concentrations of phenolic acids than tuber skin, but the major phenolic acid was again chlorogenic acid. This once more suggests that the flowers have a different pattern of regulation than tubers, with less flow into the phenolic acid pathways and more into the flavonol and flavan-3-ol pathways, rather than just the anthocyanin pathway. The explanation of why, or how, these different patterns in the regulation of the flavonoid and phenolic acid pathways between the tubers and flowers occur is not known, but Liang *et al.* (1989), in a study of phenylalanine ammonia-lyase (PAL) in bean plants found that PAL is encoded by a family of three genes. Each of these genes are expressed at different levels in roots, shoots, leaves and flowers, and it was found that each gene exhibited markedly different patterns of regulation with respect to organ specificity during normal development, and also within a single organ in response to diverse environmental stimuli. The other key enzyme of flavonoid biosynthesis, chalcone synthase (CHS), has also been found to be encoded by three different genes in *Gerbera hybrida*, which results in three different, but related, enzymatic activities, with the genes *gchs1* and *gchs2* correlated with flavonol synthesis, and *gchs1* only correlated with anthocyanin synthesis in flowers (Helariutta *et al.*, 1995). The presence of similar, multiple genes for these enzymes (PAL and CHS) in potato plants may provide an explanation for the differences in regulation of anthocyanins, flavonoids and phenolic acids observed between tubers and flowers.

3.6.2.3 Leaves

Although no anthocyanins were detected in the leaves of *S. tuberosum* cultivars, in two or three of the cultivars the visible appearance of the leaves suggested that some anthocyanin was present, because the leaves appeared to have a slight purple tinge. It is not known why anthocyanins were not detected in these leaves because, in the analysis of

some white tubers with no visible colour, some anthocyanin was detected. It is possible that the high concentrations of chlorophyll may have interfered with the extraction of anthocyanins. The flavonoid pattern in leaves was similar to that in flowers, with the Qu-glycosides 2a, 2b and rutin being the major flavonoids, however there were higher concentrations of catechin and Km-3-rut than in flowers. The leaf flavonoid pattern fell into the same two categories as the flower flavonoids with the same six cultivars having low concentrations of the Qu-glycosides 2a and 2b. Leaves contained lower concentrations of phenolic acids than tuber skin, and similar concentrations and composition of phenolic acids to that found in flowers. Therefore, the regulation of phenolic acid and flavonoid biosynthesis in leaves appeared to be similar to that found in flowers, except that no anthocyanin was produced.

3.6.3 Enzyme activities of field grown *Solanum tuberosum* tubers

The lack of correlation between the activity of PAL and the final concentrations of anthocyanins, flavonoids or phenolic acids was initially surprising because PAL is thought to be one of the major regulatory enzymes in the phenolic pathway (Hahlbrock *et al.*, 1976; Stafford, 1990). However, this discrepancy may be because field grown tubers were only taken from one sampling date, and PAL activity was likely to have been higher earlier in the season when the majority of the phenolics were synthesised, before decreasing to the levels found at the time of measurement. C4H, CHI, and DHR activities showed a good correlation with anthocyanin and phenolic acid concentrations in the skin, and CHI activity in the flesh was closely correlated with the concentration of anthocyanins and flavonoids. Activity of GT with some substrates was also correlated was the concentrations of phenolics.

Urenika appeared to have an atypically low concentration of flavonoids compared with the concentration of anthocyanins. When all the cultivars were studied, there was a slight positive correlation between the anthocyanin and flavonoid concentrations (Section 3.5.1.1). However, the flavonoid concentration found in Urenika tubers was much lower than the flavonoid concentrations found in tubers from other cultivars with a similar anthocyanin concentration as Urenika (Appendix 3). Therefore, the correlation coefficient of enzyme activities with skin flavonoids tended to be low because only five cultivars were studied and the flavonoid concentrations from Urenika tubers was atypical.

For these results of enzyme activities to be meaningful, data needs to be obtained from a larger number of samples, and also during the development of the tuber. It is thought that the often low correlations between these enzyme activities and phenolic

concentrations were because the tubers were almost mature, and enzyme levels had probably decreased to background levels, bearing little or no relation to their previous activities or phenolic concentrations.

3.6.4 Other *Solanum* species

Tubers of the other available *Solanum* species did not show the range of colours shown by *S. tuberosum* cultivars, and were mostly white or light purple. The total anthocyanin concentration in the tuber skin of the other *Solanum* species ranged from 0-300μg/gFW, compared with 0-7000μg/gFW in *S. tuberosum;* no anthocyanin was found in the flesh of the tubers of the other *Solanum* species. Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1) was identified as the major anthocyanin in the skin of all tubers of the other *Solanum* species (when present), with much lower concentrations of Mv-3-(*p*-coumaroyl-rut)-5-glu (anth-2) and Mv-3-rut. No anthocyanins other than these three were detected, suggesting that either the concentration was too low for detection, or that the number of anthocyanins was greatly reduced compared with *S. tuberosum* tubers. No Pg or Pn-glycosides were detected in these other *Solanum* species, however Pg-3-rut and Pg-3-rut-5-glu have been found in *S. phureja* (Harborne and Sherratt, 1957).

The major anthocyanin in flowers of the other *Solanum* species was Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1), with lower concentrations of four other anthocyanins also found. Similar levels were found as in *S. tuberosum* flowers. The major anthocyanin in leaves of these other *Solanum* species was either Pt-3-(*p*-coumaroyl-rut)-5-glu (anth-1) or UA1, but with only low concentrations of these being found; in contrast with *S. tuberosum* leaves where no anthocyanins were detected.

There was considerable variation among plants and species in both phenolic acid and flavonoid concentrations, but *S. tuberosum* flowers contained lower average concentrations of total phenolic acids and flavonoids, and tubers and leaves contained high concentrations of flavonoids when compared with the other eight *Solanum* species, with no other significant differences being found. *p*-Hydroxybenzoic acid was found in tubers of some of the other *Solanum* species, but not detected in any *S. tuberosum* tubers. Like *S. tuberosum* the flavonoid pattern of flowers and leaves of the other *Solanum* species fell into two categories, with all species tested, except three, having low concentrations of Qu-gly 2a and 2b, whereas the majority of flowers and leaves of *S. tuberosum* cultivars had high concentrations of Qu-gly 2a and/or 2b. Of the imported lines that also had high concentrations of Qu-gly 2a and/or 2b, [*S. sparsipilum* (SPL 3488) (but not SPL 3563), *S. speggazzinii* (SPG 3745 and SPG 3791) and *S. stenotomum*], both *S. stenotomum* and *S. sparsipilum* are believed to be ancestors to *S.*

tuberosum. The diploid species (761/1, Appendix 1, Table A1.1) that was field grown with *S. tuberosum* cultivars had very low concentrations of Qu-gly 2a and 2b, similar to most of the imported species. Because the flavonoids of the flowers and leaves of each species and/or cultivar fell into one category it means that it may be possible to distinguish different species and even different genotypes on the basis of flavonoid composition.

The Solanaceae family is known to contain a variety of flavonols and, in general, does not often contain any free flavones; however, luteolin and apigenin have been found in some *Solanum* species. The only flavone glycoside to have been isolated is luteolin-7-glucoside from petals of *S. stoloniferum* (Harborne, 1962). No flavones were identified in the *Solanum* species or *S. tuberosum* cultivars investigated in this study, however it is possible that they occurred in very small amounts and were not identified.

Wietschel and Reznik (1980b) studied leaf flavonoids in tuber-bearing *Solanum* species from the *Tuberosa* series and found that Qu-glycosides predominated, accompanied by Km-glycosides and in some instances isorhamnetin- and My-glycosides. The same pattern was found in this study with 70-85% of the flavonoids being Qu-glycosides, and 2-10% Km-glycosides, although isorhamnetin-glycosides and My-glycosides were not identified.

3.6.5 Effect of disease

The effect of late blight (*Phytophthora infestans*) on tubers of *S. sanctae-rosae* was to greatly increase the concentration of phenolic acids and flavonoids in both the skin and flesh of diseased tubers, with no increase in anthocyanin concentration. There was a hundred fold increase of flavonoids in the flesh, with epicatechin, eriodictyol and naringenin not produced in the flesh of healthy tubers, but produced in the flesh of diseased tubers. Friend *et al.* (1971) found increased chlorogenic acid and PAL activity in resistant tuber tissue which was inoculated with *Phytophthora infestans*. Lee and Le Tourneau (1958) found that potato varieties, resistant to *Verticillium* wilt, contained more chlorogenic acid than did susceptible varieties. Kuc *et al.* (1956) found that two to three times the levels of chlorogenic and caffeic acids were produced in response to inoculation by a fungus, and that these phenolic acids possessed inhibitory properties and were associated with the immunity of potatoes to the fungus. These findings were supported by Kuc (1957) in a study using a range of potato pathogens, but it was found that the production of chlorogenic and caffeic acid did not account for all the inhibitory activity to the pathogens. Geissman (1956) has reported that cherry and peach leaves infected with a virus contained two to three times the levels of *p*-coumaric acid, esters of

p-coumaric acid, Km, Qu, caffeic acid and chlorogenic acid as healthy leaves. Apple tree leaves resistant to scab (*Venturia inaequalis*) contained higher concentrations of flavonols and p-coumaric acid derivatives than susceptible cultivars (Picinelli *et al.*, 1995). However, Olsson and Jonasson (1995) found that varietal differences of chlorogenic acid in potato tubers had no effect on the susceptibility to wireworm attack. Therefore, although the presence of flavonoids and phenolic acids in the potato tuber may play a role in the defence against fungal pathogens, it is debatable whether this role includes defence against other pests.

In potatoes, as in most other plants, wounding, illumination and disease are known to stimulate various phenylpropanoid pathways (Hahlbrock and Scheel, 1989). In this study of diseased tubers, there was increased output through the cinnamic acid, benzoic acid and flavonoid, especially flavonol, pathways. It is interesting that no flavonols were detected in healthy tubers but, in diseased tubers low concentrations of three flavonols were detected, as well as increased levels of the other flavonoids. Therefore, the additional flow through the flavonoid pathway went along the previously unused flavonol pathway instead of the anthocyanin pathway in which the enzymes were presumably already active. This suggests that the potato tubers contained the genes for these flavonoid pathways, even though in the normal pattern of growth they were not transcribed.

These results support the theory (Hahlbrock, 1981; Grayer, 1989; van Sumere, 1989; Weidenbörner and Jha, 1993) that one of the reasons the potato produces phenolic acids and flavonoids is to aid in plant defence. Also supporting this hypothesis is the fact that much higher concentrations of phenolic acids and flavonoids (and anthocyanins) were found in the skin of the tuber rather than in the flesh, and this may be related to the skin being the first line in the defence of the tuber against pathogens and pests.

CHAPTER 4

Changes in anthocyanin, flavonoid, phenolic acid and carotenoid concentrations during tuber development and storage

4.1 Introduction

4.1.1 Changes in tuber colour

Generally, when consumers purchase potatoes they are concerned about the colour of the tubers as well as any changes which may occur before they eat them. The grower and marketer are also interested in changes which may occur between the time of harvest and the time of sale. However, from the biosynthetic point of view it is important also to determine the developmental regulation of anthocyanins in the tuber. There has been little research carried out into the changes of flavonoid and anthocyanin levels during the development of tubers or their metabolism during storage. The production of colour in tubers maybe caused by the accumulation of anthocyanin pigments during growth and/or storage, and/or a change in the physiochemical environment of the pigments, resulting in a colour change even though no quantitative change in anthocyanin occurs. This change in physiochemical environment may be caused by the production of other phenolics which can act as copigments, a change in vacuolar pH, or the presence of other compounds, such as metal ions that may have an effect on the expression of anthocyanin colour. These and other factors have been discussed in Chapter 2.

Changes in anthocyanin concentration during development, and hence the final colour, may be influenced by the environmental and growth conditions experienced by the plant and tuber, such as water potential, temperature, disease, nutrient levels and other nutritional factors. Similarly, the resultant colour after storage may depend on factors including handling during harvest, storage conditions and the length of storage time. Damage to the tubers during harvest, exposure to light and the presence of pathogens are particularly important because these factors are known to increase the activity of the enzyme phenylalanine ammonia-lyase (PAL), and the concentrations of phenolic acids, flavonoids and anthocyanins, and may alter the tuber colour.

Note that throughout this chapter, the term "flavonoid" refers to all the classes of flavonoid compounds except for the anthocyanins, as these are reported and discussed separately from the other flavonoids.

1) to determine at what stage of tuber development the anthocyanins (and also other flavonoids and phenolic acids) are produced, and to monitor any changes in anthocyanin, flavonoid and phenolic acid concentrations throughout development,

2) to determine the distribution of anthocyanin within the tuber at various stages of development and after storage, and

3) to evaluate the effects of storage of different lengths of time, and in different conditions, on the anthocyanin concentration.

Thus, it was envisaged that some insight into the regulation of anthocyanin biosynthesis during the development of the tuber would be gained, as well as to determine the optimum time of harvest and storage conditions for maximum anthocyanin colour retention for the consumer. The carotenoid concentration and changes during tuber development were also monitored to determine if this other major tuber pigment followed similar changes as the anthocyanins, and whether the carotenoids had any effect on overall tuber colour.

4.1.2 Formation and development of tubers

Tuber formation begins in the sub-apical region of the rhizome in the youngest elongating internode, and is first visible externally as a radial enlargement of this region (Cutter, 1978). This is due initially to the increase in cell size in the pith region, with subsequent rapid increases in cell division and further cell enlargement. After the initial expansion of this single internode, internodes closer to the apical meristem also begin radial enlargement leaving the rhizome apex at the end of the tuber. Very little extension growth occurs in the most apical internodes so that they remain very short, and therefore in the mature tuber the nodes are closely spaced at the apical (bud) end (and become the "eyes"). The final tuber size is achieved primarily though an increase in cell volume, particularly in the pith and inner cortical parenchyma cells (Peterson *et al.*, 1985).

The shift in the ratio of the plant hormones abscisic acid (ABA) : gibberellic acid (GA) is considered to be one of the key factors in controlling tuberization. Environmental factors, particularly photoperiod, temperature and nitrogen nutrition, are also involved in control by altering the levels of these hormones (Krauss, 1985).

4.1.3 Storage

Mature tubers are generally dormant for the three to four months after harvest, before sprouting occurs. The concentration of major constituents (carbohydrates, protein, nucleic acids, etc.) reaches a steady level before the tuber is harvested. During storage,

nucleic acids, etc.) reaches a steady level before the tuber is harvested. During storage, the concentration of most of these constituents remains relatively constant. Even though there is a perpetual cycling of some constituents, the breakdown and production is nearly in balance, except for the loss of carbon from the system in the CO_2 evolved in respiration. Mature tubers have a low rate of respiration which decreases slightly during storage until the start of sprout growth, when it rises progressively (Dizengremel, 1985).

Under good storage conditions the percentage of dry matter (%DM) is expected to remain relatively constant with only a 0.1% drop per month (Burton, 1978). This is because, although dry matter is being lost by respiration, water loss during storage is also occurring and the two balance out. However, if the temperature is increased, although the respiration rate will rise, water loss is increased to a greater extent, causing an increase in %DM; similarly at low humidities, the increased rate of water loss also causes increased %DM during storage (Scott and Wilcockson, 1978).

The change in sugar concentration in the tubers during storage may be an important determinant for anthocyanin pigmentation because the concentration of sugars has been found to influence the production of anthocyanins (see Section 4.1.4). The carbohydrate reserves in tubers are extremely responsive to the environment, and starch is converted to high concentrations of sugars as a result of stresses experienced during storage (e.g. handling, anerobiosis, exposure to high or low temperatures, ageing or senescence) (Sowokinos, 1990a and b). If tubers are maintained at a constant temperature from the time of harvest (and suffer from no other stresses), there would be no marked change in sugar content until after a few months when the tubers would begin to "sweeten". The starch is converted to sugars, and the timing and degree of this sweetening parallels the sprout growth. This phenomenon, called "senescent sweetening", is linked to changes in the membrane surrounding the starch grains, and is irreversible (Isherwood, 1976). Sweetening also occurs after the storage of tubers at low temperatures (e.g. 2°C), however this "low temperature sweetening" differs from "senescent sweetening" in being reversible, to a large extent, if the temperature is raised, and the sugars may be converted back to starch (Burton, 1978).

No mention of any change in flavonoid levels during the storage of tubers could be found in the literature, although the phenolic acid, chlorogenic acid, has been found to increase in the buds during storage (Burton, 1978). Similarly, there were no references to changes in anthocyanin concentration in tubers during storage however, Verma *et al.* (1972) investigated the differences in pigments of light and dark grown sprouts, and Houwing *et al.* (1986) used sprout colour as a characteristic to aid in the identification of different cultivars. The anthocyanin and flavonoid changes in tuber sprouts were not

investigated in these experiments, because only the final colour of tubers for consumption and the associated biochemistry was of interest, and therefore studies were limited to only tuber tissue.

4.1.4 Effect of carbohydrates

Anthocyanin accumulation is generally thought to be stimulated by sugars. Eddy and Mapson (1951) found that the anthocyanin concentration of cress seedlings increased when exogenous glucose, fructose, sucrose, sorbose, galactose or arabinose was fed, and that the anthocyanin content was related to the total sugar content rather than to the concentration of any one sugar. They concluded that the effect of the sugar was indirect, possibly because of effects on general metabolism. A similar finding was made by Thimann *et al.* (1951) using *Spirodela oligorhiza.*

In apple peel, various treatments known to increase the activity of the pentose phosphate pathway were correlated with increased anthocyanin accumulation (Faust, 1965). The "spring flush" of anthocyanin colour in the young leaves of many plants and the production of autumnal reds in dying leaves has been attributed to the fact that in both these situations, the leaves are particularly rich in free sugars (Harborne, 1967), and it is thought that the excess sugar upsets the metabolism in the direction favouring anthocyanin synthesis, to prevent excessive accumulation of free sugar in the cells.

4.1.5 Determination of the surface area of tubers

In the first season (1992/93), only tubers greater than 5g were analysed because it was difficult to peel tubers smaller than this size and determine the weight of the coloured skin. For the second season (1993/94), a method was developed to enable these smaller tubers (<10g) to be analysed. Therefore, in the analysis of these small tubers it was decided to use the surface area (SA) so these tubers could be compared with each other. It was considered that the shape of a typical tuber approximated that of an ellipsoid (McRae *et al.*, 1986; Meredith, 1989; 1995), so the formula to determine the surface area of an ellipsoid was used to calculate the approximate surface area of the tubers.

For calculations, "l" and "w" were defined as the length and width of the tuber, respectively. When $l > w$, as in the case of the potato, the surface area of an ellipsoid can be determined as follows:-

$$SA = 2\pi w^2 + (2\pi l^2 w) / \sqrt{(l^2 - w^2)}\ \sin^{-1}[\sqrt{(l^2 - w^2)} / l]$$

If the eccentricity is defined as:-

$$e = \sqrt{(l^2 - w^2)} / l$$

then $SA = 2 \pi w^2 + (2 \pi l w) / e \sin^{-1}[e]$

When $l = w$, as in the case of a perfectly round tuber, which sometimes occurred in smaller tubers, using an ellipsoid to approximate the shape was no longer valid, and instead the tuber shape was approximated by a sphere. In these cases the surface area was calculated by $SA = 4 \pi r^2$, where "r" equals ½ l (or ½ w), so the formula became:-

$$SA = \pi l^2$$

4.2 Materials and methods

4.2.1 Plant material

4.2.1.1 Tuber development

Field grown tubers of Arran Victory, Desirée, Red Flesh and Urenika cultivars were grown at Lincoln by the NZ Institute for Crop & Food Research Ltd. All the tubers from one plant of each cultivar were sampled at weekly or fortnightly intervals throughout tuber development, starting as soon as the tubers reached over 5g, continuing until no further increase in tuber weight was observed. The dates of sampling for the 1992/93 season were 7/1, 27/1, 15/2, 1/3, 15/3, and 29/3/93. For the 1993/94 season the dates of sample collection were 12/1, 19/1, 26/1, 2/2, 9/2, 16/2, 2/3, and 23/3/94. Tubers were divided into size classes based on weight as shown in Table 4.1.

At each sampling date, all the tubers were removed from the plant and washed and weighed. For the 1992/93 season only, tubers over 5g were analysed, because smaller tubers were too small for analysis. For analysis of the tuber skin, the skin was peeled carefully from the tubers, with care taken to remove all flesh, and the skin sample was weighed. For tubers less than 50g in weight all the skin from the tuber was used, but for tubers larger than 50g a representative portion of skin was taken from the centre of the tuber, between the stem and bud ends (because the distribution of anthocyanin from one end of the tuber to the other was found to vary (Section 4.4.4)). For analysis of tuber flesh, all the skin was removed and a representative slice of the flesh at the centre of the tuber was taken, and the flesh sample was weighed. Samples were up to 2g in weight. Only skin samples were analysed for Arran Victory and Desirée tubers because they

contained anthocyanin in the skin only, but both the skin and flesh were analysed for Red Flesh and Urenika tubers, which contained anthocyanin throughout the tuber.

In the 1993/94 season, all tubers (over 2mm diameter) from the plant were sampled because the calculation of the surface area, rather than fresh weight (FW) enabled the smaller tubers (<10g) to be analysed. For tubers under 10g, tubers were weighed, the length and width of the tuber measured, and the whole tuber analysed, whereas tubers over 10g were assayed as for the first season. Additionally, for the second season, tubers were cut into two approximately equal halves (stem end and bud end) which were analysed separately for anthocyanins (and for other flavonoids and phenolic acids where measured) to determine if there was any difference in the concentration of phenolics between each end of the tuber.

Table 4.1 Size classes (g) used for analysis of tubers during development.

lower limit	upper limit
0.0	0.09*
0.1	0.29*
0.3	0.49*
0.5	0.69*
0.7	0.99*
1.0	2.99*
3.0	4.99*
5.0	9.99
10.0	19.99
20.0	29.99
30.9	49.99
50.0	69.99
70.0	99.99
100.0	149.9
150.0	199.9
200.0	249.9
250.0	299.9
300.0	399.9

* 1993/94 season only

4.2.1.2 Storage

Arran Victory, Desirée, Red Flesh and Urenika tubers from the 1992/93 season were harvested after shoot senescence on 10/6/93, and stored at 4°C with 86% humidity, immediately after harvest. Random samples of five tubers from each cultivar were taken at the following dates:- 10/6 (harvest), 23/8, 8/10, 16/11, and 22/12/93, the phenolics extracted and the anthocyanin concentration measured using the Hewlett Packard 8452A diode-array spectrophotometer. Additionally, Desirée extracts were analysed by analytical HPLC for individual anthocyanin, flavonol and phenolic acid concentrations. For the 1993/94 season, tubers of the same four cultivars were harvested on 1/6/94 and stored at 4°C with 86% humidity. Tubers were weighed at harvest and again when sampling after storage. Random samples of five tubers of each cultivar were taken at fortnightly intervals from the harvest date (1/6/94) to the final sampling date (30/11/94). Anthocyanins were extracted from portions of the skin and/or flesh as in Section 4.2.1.1 and their concentration recorded. Remaining portions of each tuber, not used for anthocyanin extraction, were freeze-dried and the %DM content calculated. For the 1993/94 season, the effect of storage temperature on the anthocyanin concentration of stored tubers was also investigated. Tubers were kept outside (0-10°C) for six weeks after harvest before the experiment was carried out, and then stored at each of four temperatures: 4°, 10°, 18° and 26°C, with the respective humidities at each temperature being 86%, 55%, 46%, and 32%. Anthocyanin concentrations from five randomly selected tubers at each storage temperature were measured at fortnightly intervals for ten weeks.

4.2.1.3 Distribution of anthocyanins within the tubers

The distribution of anthocyanin (as a concentration in the different parts of the tuber) was measured in mature, stored tubers of Arran Victory, Desirée, Red Flesh, and Urenika, with tubers sampled after four months storage at 4°C. The distribution of anthocyanins in Desirée skin was also measured for tubers during development (where plants were harvested during the period where tubers were still increasing in size), and tubers in the size classes of 50-70g and 70-100g were analysed. Each tuber was cut into five approximately equal slices and the anthocyanin concentration analysed by spectrophotometry. For comparison with measurements of the anthocyanin concentration by extraction and spectrophotometric measurement, the distribution of pigment in the flesh of Urenika tubers was also measured by image analysis. Tubers were cut into five slices as before and the colour density of each slice measured across the cut surface of the slice using the image analyser at the NZ Institute for Crop & Food

Research Ltd, Lincoln, following the method of Coles *et al.* (1993), except that slices were measured individually (rather than in groups).

4.2.2 Anthocyanin, flavonoid and phenolic acid analysis

Phenolics were extracted in 15% acetic acid in methanol as outlined in Section 3.3.2. In most cases the concentration of anthocyanins was measured spectrophotometrically. A known volume of extract (5-300µl) was added to 200µl 1% HCl in methanol, and made up to 500µl with 15% acetic acid in methanol. The spectrum was recorded on the diode-array spectrophotometer, and the absorbance at λ_{max} determined. The absorbance at 700nm was subtracted to correct for any turbidity, because the anthocyanins had no absorbance at 700nm. The anthocyanin concentration in Desirée and Red Flesh samples was calculated from a Pg-3-rut calibration curve, and for Arran Victory and Urenika extracts a Mv-3-rut calibration curve was used (see Section 4.3.3). Additionally, for Desirée samples the identification and quantification of anthocyanins, flavonoids and phenolic acids in skin samples from both seasons throughout development, and the storage for the first seasons tubers, were carried out by analytical HPLC, as in Section 3.3.3.2. This was carried out to identify and monitor the changes in the individual anthocyanins, flavonoids and phenolic acids, and also as a comparison of total anthocyanins between the two methods. HPLC analysis could not be used for all cultivars because it was too time consuming and expensive.

4.2.3 Carotenoid analysis - extraction and quantification

Developing tubers of Arran Victory, Desirée, Red Flesh and Urenika from the 1992/93 season were used for assay of carotenoid concentrations. Approximately 2g of tuber tissue was ground in liquid N_2, and the resulting fine powder extracted three times in a total of 25ml cold acetone, which was filtered through Miracloth and centrifuged at 12 000g, for 10min at 4°C. The supernatant was evaporated to dryness *in vacuo*, and the carotenoids redissolved in 0.9ml 80% acetone / 20% water. Hexane (1.35ml) was added and, after thorough mixing, the two phases allowed to separate. The spectrum of the top hexane fraction, containing the carotenoids, was recorded using the diode-array spectrophotometer. The absence of absorbance owing to anthocyanins (500-550nm) and chlorophyll (640-680nm) in the spectrum was confirmed, and the absorbance of carotenoids at 446nm was recorded. Because there was no absorbance due to carotenoids at 550nm, the absorbance at 550nm was subtracted to correct for turbidity, and the carotenoid concentration calculated from a calibration curve of β-carotene absorbance.

4.3 Evaluation of methods

4.3.1 Extraction of phenolics

4.3.1.1 Extraction solvent

Tuber tissue was extracted with one of either:- 15% acetic acid, 1% HCl or 1% trifluoroacetic acid (TFA), each in methanol, and the extracts analysed by analytical HPLC. The samples extracted with the HCl or TFA solvent contained only about 10% of the concentration of chlorogenic acid that was present in the acetic acid extracted samples. Because these two samples also contained higher concentrations of caffeic acid, it was thought that the use of these strong acids caused the hydrolysis of chlorogenic acid (probably at the stage when samples were dried using a rotary evaporator, and the acidic extract was heated and concentrated at 40°C). Although in these experiments the concentrations of other phenolic acids and flavonoids appeared similar to those in the acetic acid extract, other workers have found that stronger acids often cleave acyl groups from flavonoids (Strack and Wray, 1989), and therefore 15% acetic acid in methanol was used as the extraction solvent in all experiments.

4.3.1.2 Extraction efficiency

The number of extractions required for the complete recovery of anthocyanins was investigated. Triplicate samples of Red Flesh skin and Urenika flesh (0.5g) were ground in liquid N_2 and extracted six times with 10ml 15% acetic acid in methanol. Each separate extract was filtered, centrifuged (12 000g, 10min, 4°C), evaporated to dryness *in vacuo* and redissolved in 1.5ml 15% acetic acid in methanol, and the anthocyanin concentration measured by spectrophotometry, as in Section 4.2.2.2.

Table 4.2 Extraction efficiency of anthocyanins from skin (Red Flesh) and flesh (Urenika) samples (se = standard error).

Extraction number	% of total anthocyanins extracted			
	Red Flesh	se	Urenika	se
1	89.7	2.2	87.9	1.8
2	96.4	2.2	96.0	1.9
3	98.3	0.4	98.7	0.3
4	99.5	0.3	100	0.2
5	99.8	0.3	100	0
6	100	0.2	100	0

After four extractions, 99.5% of the anthocyanins in the Red Flesh skin samples was extracted, whereas for the Urenika flesh samples extraction was complete (Table 4.2). Extraction was also tested by spiking an initial sample of a white tuber (which contained no absorbance due to anthocyanins) with known concentrations of Cy-3-glu. Over 99.5% of the standard was recovered after two extractions. In general, isolation of anthocyanins from skin tissues required more extractions, which is probably because of the cell structure and the presence of waxes in the skin. Therefore, in all future extractions, tissue was extracted four times and these extracts pooled.

4.3.2 Stability of anthocyanins

Extracted anthocyanins from Red Flesh skin and Urenika flesh were stored at -18°C and the absorbance of the anthocyanins measured at intervals over eight months. There was no decrease in anthocyanin absorbance during this period, and therefore it was assumed that no degradation of anthocyanins had occurred. Therefore, in further experiments, the analysis of anthocyanins by spectrophotometry was carried out within two days of extraction, but the analysis by HPLC was often not performed for two to three months.

4.3.3 Calibration curves for anthocyanin concentrations

To calculate the concentration of anthocyanin present in samples, two calibration curves were prepared (with triplicate readings of the standard at ten different concentrations): 1) Pg-3-rut, used for calculation of the anthocyanin concentration of Desirée and Red Flesh samples, and 2) Mv-3-rut, for Arran Victory and Urenika extracts. Both standards gave a linear calibration curve up to 20μg/ml with R^2 values of 99.4% and 99.9% respectively. The equations of each line were

Pg-3-rut concentration (μg/ml) = (15.958 *x* absorbance) + 0.124

and Mv-3-rut concentration (μg/ml) = (13.193 *x* absorbance) - 0.028.

4.3.4 Measurement of anthocyanin absorbance and effect of pH

The effect of pH on anthocyanin colour is well known, with increased acidity causing an increase in absorbance. The colour of potato anthocyanins in 15% acetic acid in methanol was low because of the weak acid. It was not always possible to remove all traces of acetic acid when the sample was dried *in vacuo*, so after being redissolved in 15% acetic acid, the pH of the sample may have varied slightly. 1% HCl in methanol was not used for the extraction of phenolics because it was found to hydrolyse chlorogenic acid (Section 4.3.1.1) however, to increase the anthocyanin absorbance and

provide a more consistent pH when anthocyanin concentrations were measured by spectrophotometry, 1% HCl in methanol, to a final concentration of 0.6%, was added before measurement of anthocyanins.

4.3.5 Extraction of carotenoids

The number of extractions required for the complete extraction of carotenoids was investigated. Triplicate samples of Desirée flesh (1.5g) were ground in liquid N_2, and extracted four times with acetone. Each separate extract was filtered, centrifuged (12 000g, 10min, 4 C), evaporated to dryness *in vacuo*, and the carotenoids redissolved in 80% acetone / 20% water and assayed as above (Section 4.2.3). The carotenoids were completely extracted from the potato tissue after two extractions (Table 4.3), but three extractions were used in all further experiments.

Table 4.3 Extraction efficiency of carotenoids (se = standard error).

Extraction number	% of total carotenoids extracted	
	mean	se
1	87.1	2.6
2	100	2.6
3	100	0
4	100	0

4.3.6 Stability of potato carotenoids

Carotenoids were extracted in acetone and stored at -18 C, and the absorbance at 446nm measured at intervals over five days. The carotenoid absorbance remained at 100% for the first four hours, after which it began to decrease slowly. At the end of five days, 75% of the carotenoids remained. Therefore, in all further experiments the analysis of carotenoids was carried out within two hours of extraction, to minimise any loss of carotenoids.

4.3.7 Analysis of carotenoids

At the beginning of this thesis it was envisaged that some work would be carried out on the light induction of carotenoids, and comparison of the biochemistry of this class of compounds with the anthocyanins and flavonoids. However, the project was narrowed

down to include only anthocyanins, flavonoids and the related phenolic acids, with only a limited study of the carotenoids. The concentration of carotenoids and changes throughout the development of the tubers in Arran Victory, Desirée, Red Flesh and Urenika cultivars were investigated. Therefore, a quick method was required for the quantitative analysis of carotenoids. The spectrum of potato carotenoids showed a peak at 446nm (Figure 4.1), however in coloured potatoes anthocyanins were also present, which also absorbed at 446nm and interfered with a simple spectrophotometric assay at this wavelength.

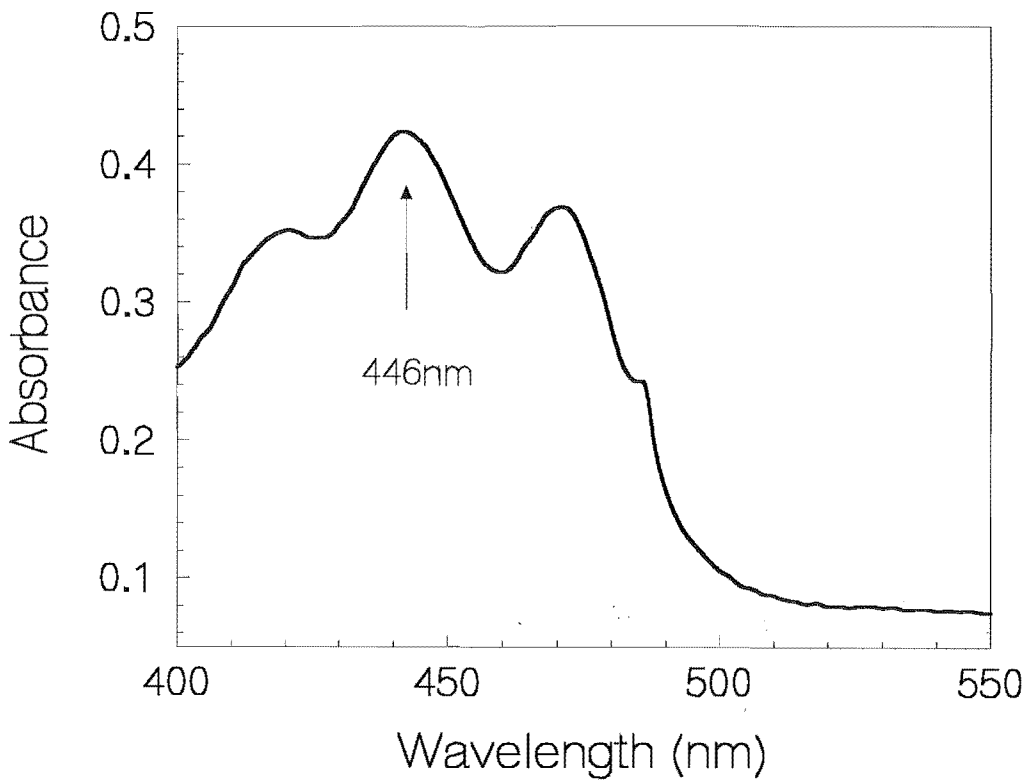


Figure 4.1 Spectrum of potato carotenoids.

Mild alkali (0.1M-NaOH) was added to the acetone extract to decolourise the anthocyanins, but alkali also caused a bathochromic shift in other flavonoid spectra, producing a bright yellow colour which absorbed strongly at 446nm. When re-acidified, this yellow colour disappeared but the anthocyanin colour returned. Therefore, there was no permanent discolouration of anthocyanins in mild alkali. Stronger alkali (1M-NaOH) resulted in the irreversible degradation of the anthocyanins, and the formation of brown coloured degradation products, which also interfered with the carotenoid assay.

A commonly used method for purifying carotenoids is that of Davies (1976), which involves the partitioning of carotenoids with hexane and ammonium sulphate, followed by the precipitation of impurities with 25% (w/v) KOH in methanol. This method was investigated and appeared to produce pure carotenoids however, after the hexane/ ammonium sulphate steps 72.4% of the carotenoids remained, whereas after the KOH precipitation step only 23.0% of the original carotenoids remained. Therefore, this was not a useful method for the quantitative measurement of carotenoids in potatoes.

The partitioning of carotenoids into hexane was investigated further, and addition of hexane to the carotenoid extract in 80%(aq) acetone caused the partitioning of the carotenoids into the hexane fraction leaving the anthocyanins in the acetone fraction. It was found that the optimum amount of hexane to use was 1.5 times the volume of acetone (Figure 4.2), however a small amount of carotenoids remained in the acetone fraction (approximately 7%). Nevertheless, hexane partitioning was found to be the optimal method to separate carotenoids from anthocyanins for this project. (Note that if chlorophyll is also present in the extract, an alternative method of carotenoid purification is necessary because chlorophyll also absorbs at 446nm and is partitioned into the hexane fraction. However, this was not necessary for these samples as chlorophyll was only present in light exposed tubers, and was not present in tubers grown and stored in the dark.)

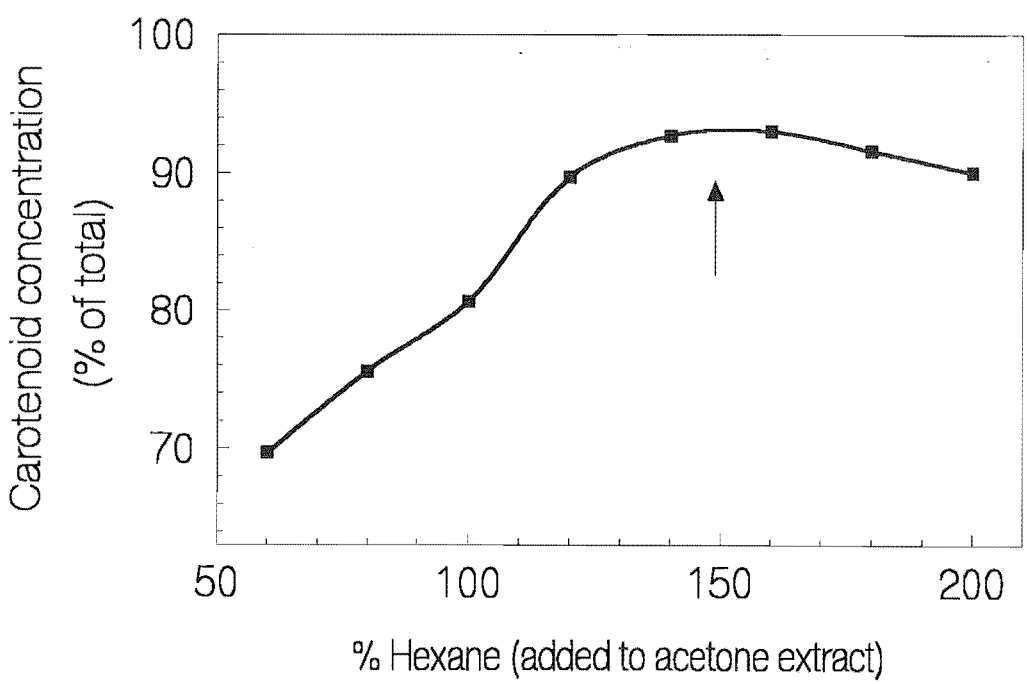


Figure 4.2 Amount of hexane to add to acetone extract for effective extraction. Arrow represents the optimum amount of hexane to add.

4.4 Results

4.4.1 Levels of phenolics in developing tubers

4.4.1.1 1992/93 Season

During the 1992/93 season, plants of Arran Victory, Desirée, Red Flesh and Urenika were harvested at fortnightly intervals during the period of tuber development, and the concentrations of anthocyanins measured in the skin and/or flesh. Additionally, for Desirée tubers the concentrations of flavonoids and phenolic acids were recorded. Although only one plant was harvested at each time and was not sufficient for statistical analysis, there were between 5 and 25 tubers used from each plant (depending on cultivar and time of harvest), and these results did enable the general trends to be detected.

Within any one cultivar, tubers of a similar size had similar concentrations of anthocyanin, and this was independent of the sampling date (*i.e.* independent of the age of the plant). This was also found for the flavonoid and phenolic acid concentrations in the skin from Desirée tubers. Because there was no effect of sampling date, the data from tubers of a similar size were grouped together for further analysis of results, so that a statistical analysis could be carried out on this larger sample.

The anthocyanin concentration in the skin of Desirée tubers (Figure 4.3a) and Arran Victory tubers (not shown) both showed similar changes with increasing tuber weight. In Desirée skin samples, the anthocyanin (Figure 4.3a) and flavonoid (Figure 4.3b) concentrations increased with increasing tuber weight, reaching a maximum (of about 1750μg/gFW anthocyanins and 550μg/gFW flavonoids) in tubers weighing between 150 and 200g, however then decreased in tubers weighing between 250 and 400g. There was a strong positive linear correlation between anthocyanin and flavonoids concentrations, with a correlation coefficient of 91.9%. The phenolic acid concentration in the skin of Desirée tubers (Figure 4.3c) showed a similar pattern to anthocyanin and flavonoid concentrations with increasing tuber weight, except that the phenolic acid concentration (of about 5100μg/gFW) peaked at a slightly lower weight, in tubers weighing between 70 and 100g, then decreased in concentration in tubers weighing between 150 and 400g.

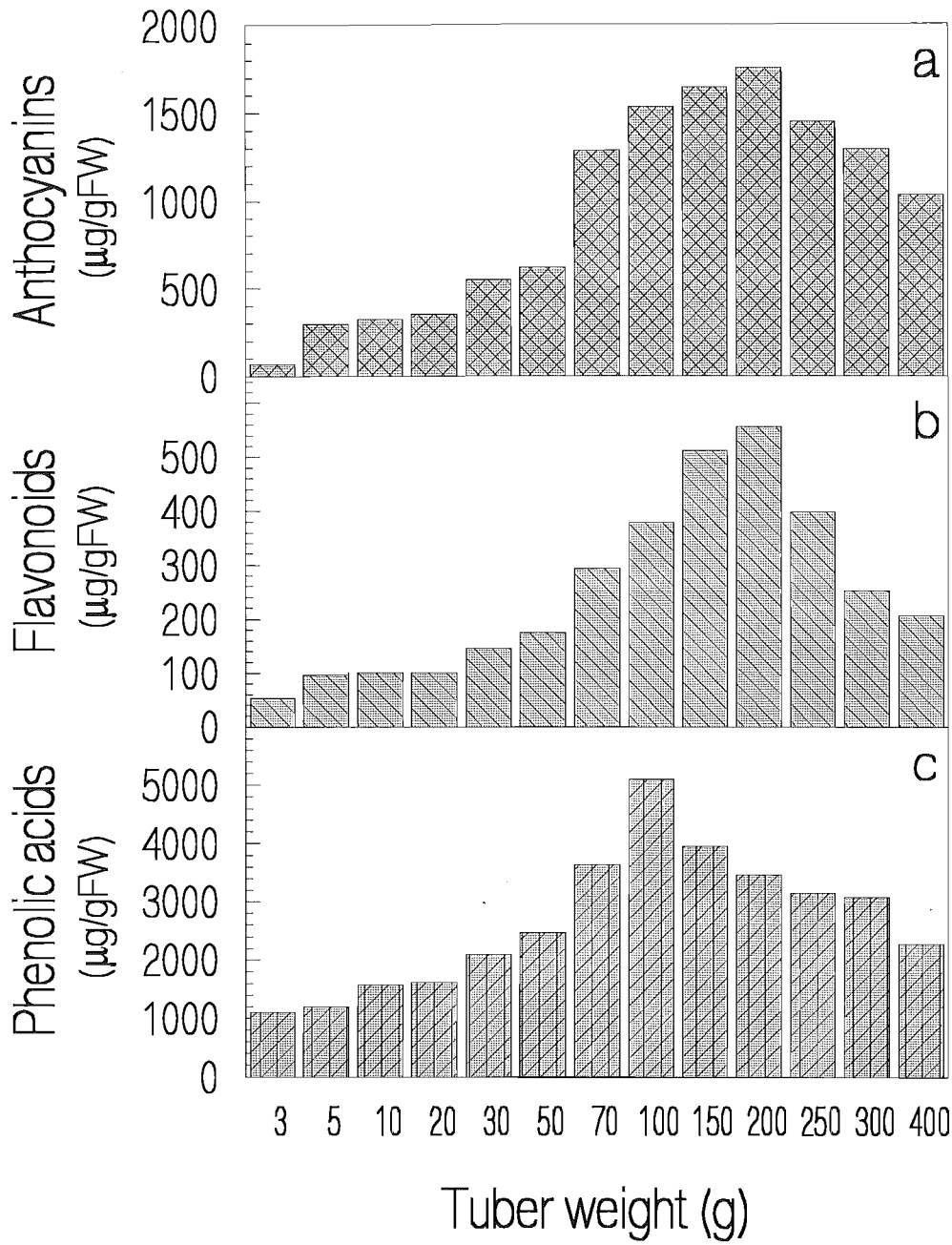


Figure 4.3 Concentration of a) anthocyanins, b) flavonoids, and c) phenolic acids in Desirée skin samples averaged over the 1992/93 season.

As indicated previously (Chapter 3), the major anthocyanin in the skin of Desirée tubers was Pg-3-*p*-coumaroyl-rut-5-glu (anth-3), with other anthocyanins found at much lower concentrations. All anthocyanins showed a similar proportional increase in concentration (Figure 4.4), with a similar percentage of the different anthocyanins found throughout the development of the tuber. Similar patterns were found with flavonoid and phenolic acid concentrations, where the proportions of individual compounds within each class also remained similar.

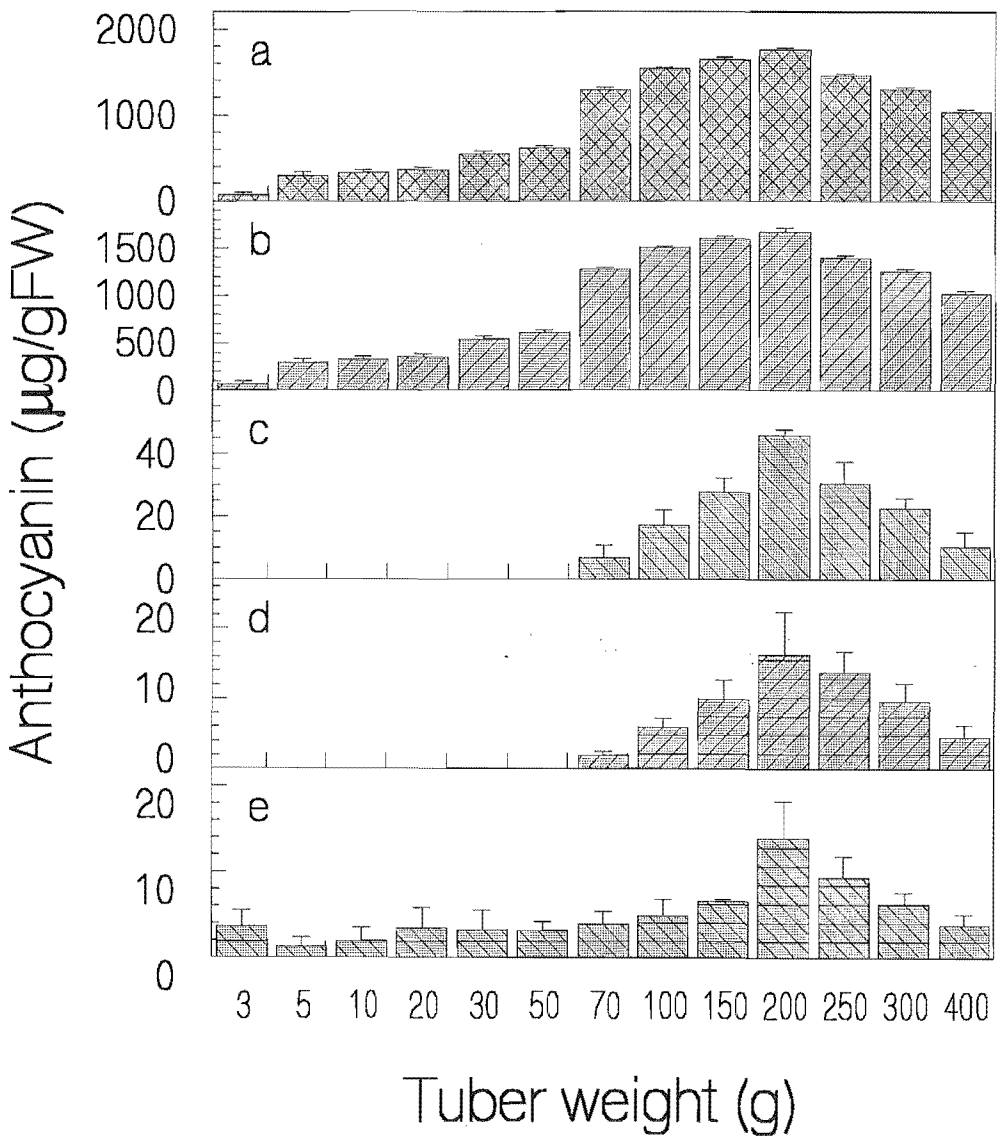


Figure 4.4 Changes in concentration of individual anthocyanins during development of Desirée tubers. a = total anthocyanin concentration, b = pelargonidin-3-(*p*-coumaroyl-rutinoside)-glucoside (anth-3), c = peonidin-3-(*p*-coumaroyl-rutinoside)-glucoside, d = UA2 (pelargonidin-glycoside), and e = pelargonidin-3-rutinoside. Error bars represent ± 1 standard error.

4.4.1.2 1993/94 Season

During the 1992/93 season (in which only tubers greater than 5g were analysed), it was noticed that the biggest changes in colour occurred in very small tubers (<5g), and that the smallest tubers of Arran Victory and Desirée were often without colour. Because large changes in anthocyanin concentration were occurring in these small tubers it was obviously important to measure the anthocyanin concentration in these tubers (<5g) as well, and this was carried out in the 1993/94 season by expressing the data on a surface area basis rather than a fresh weight basis. This was especially useful for anthocyanin concentrations in Desirée tubers because, in this cultivar, anthocyanin was only found in the skin with none being detected in the flesh.

Figure 4.5 Polarity of anthocyanin formation in a Desirée tuber (20mm long) showing the coloured stem end and white bud end.

It was also observed that the distribution of pigment was not uniform over the tuber, and anthocyanins appeared to form showing polarity of distribution from the stem end to the bud end (Figure 4.5). Typically, small Desirée tubers failed to produce anthocyanin pigmentation, and the colour then formed initially from the stem end as tubers grew, and appeared to "travel" to the bud end until the whole tuber was pigmented (Figure 4.6).

However, the bud end of some tubers remained white until tubers were quite large (up to about 50g) (Figure 4.7). A similar pattern was found in Arran Victory tubers (Figure 4.8).

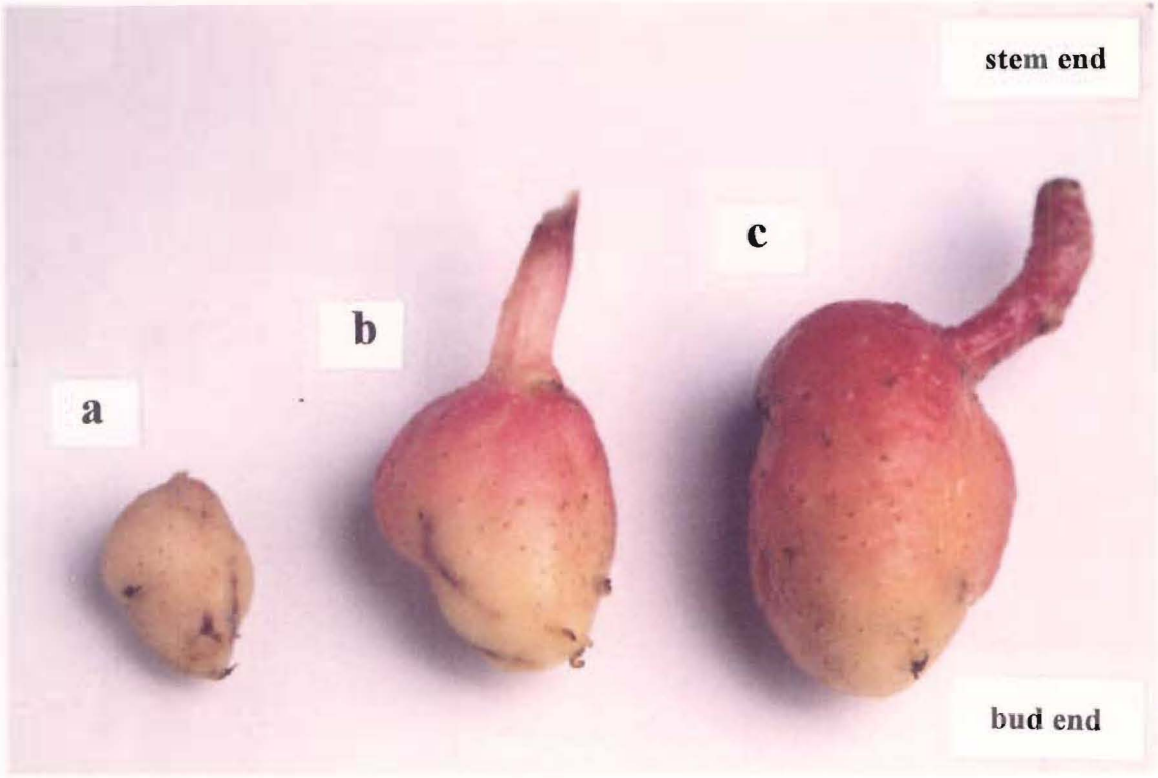


Figure 4.6 Desirée tubers showing the formation of anthocyanin from the stem end. a) 14mm long with no anthocyanin present, b) 20mm long (excluding rhizome) with the stem end half of the tuber showing pigmentation, but the bud end half remaining white and, c) 26mm long (~50g) showing intense colouration at the stem end, a lighter pink in the middle section, which gradually fades to white at the bud end.

The anthocyanin concentration in the skin of Urenika tubers increased very quickly coming to a maximum (at about 5000μg/gFW) before tubers reached 5g in size (not shown). There was also an increase in anthocyanin concentration with increased weight in the flesh of the smaller Urenika tubers (from 1g to about 20g), although anthocyanin concentrations were variable. However, the flesh of larger Urenika tubers (>50g) showed no change in anthocyanin concentration with increased size (remained at about 2000μg/gFW). There was no change in anthocyanin concentration in Red Flesh skin extracts (remained at about 3000μg/gFW) however, Red Flesh flesh samples increased to a maximum at about 20g (concentration about 100μg/gFW) (not shown).

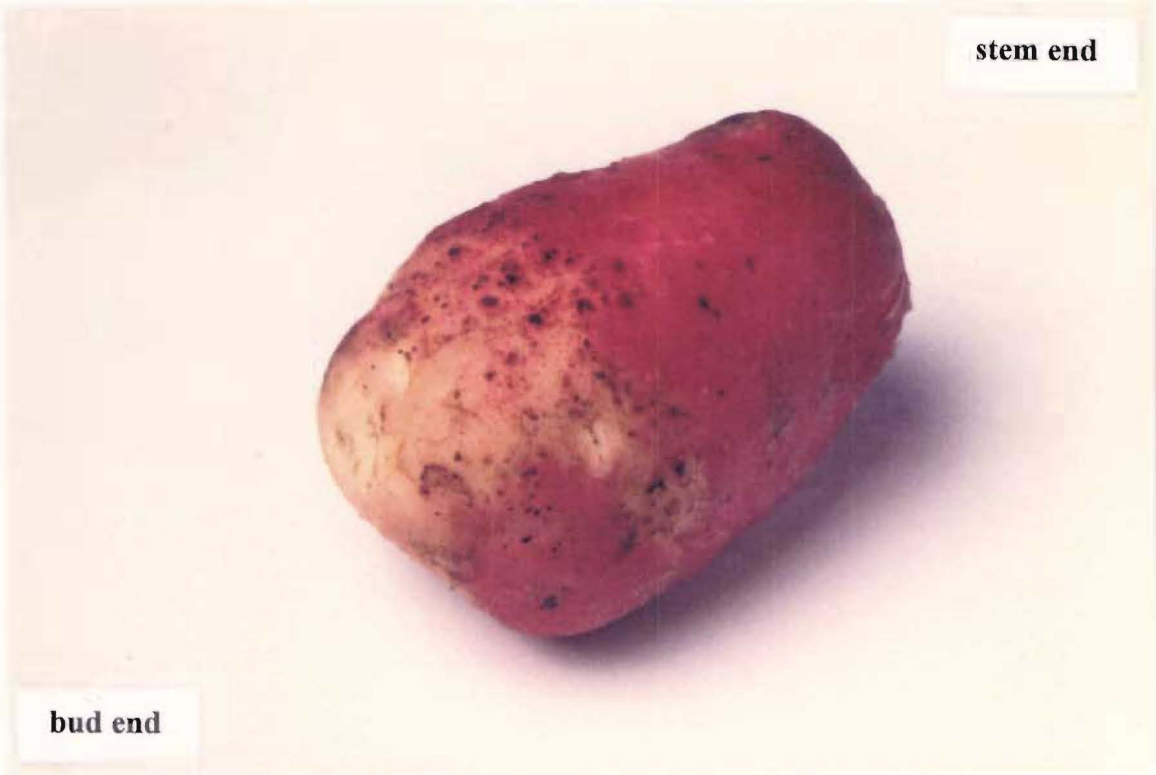


Figure 4.7 Desirée tuber (~70mm long) showing that the bud end of tubers often remained white.

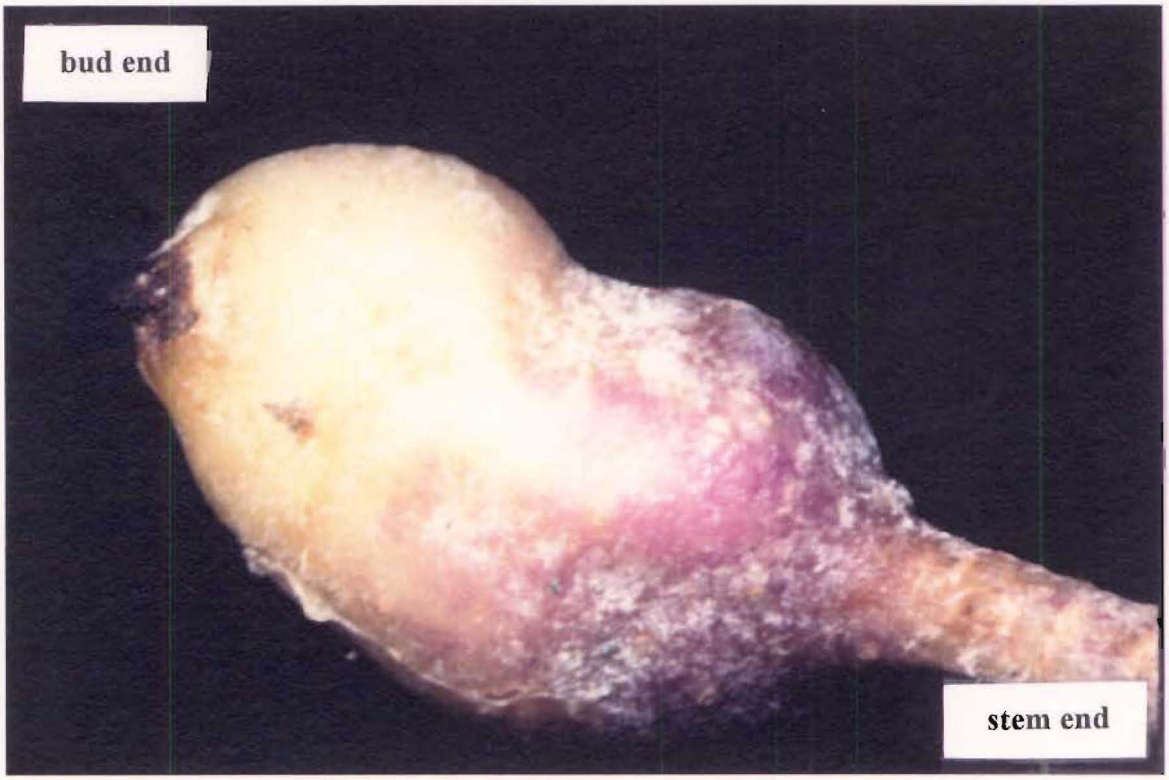


Figure 4.8 Arran Victory tuber (~25mm long) showing the formation of anthocyanin from the stem end.

In microscope studies of developing Urenika tubers, the rhizomes were very white, and a small amount of purple colour was found just behind the rhizome tip before the radial expansion of the tuber began (Figure 4.9a). As the tuber expanded, the colour of the pigment intensified, and pigment was observed further towards the bud end (Figure 4.9b to Figure 4.9d), until the typical dark purple colour was formed (Figure 4.9e). The pigment was found only in the skin, with the flesh remaining white, until tubers reached approximately 5-10mm in diameter, with anthocyanin in the flesh then gradually formed as tubers grew (Figure 4.10).

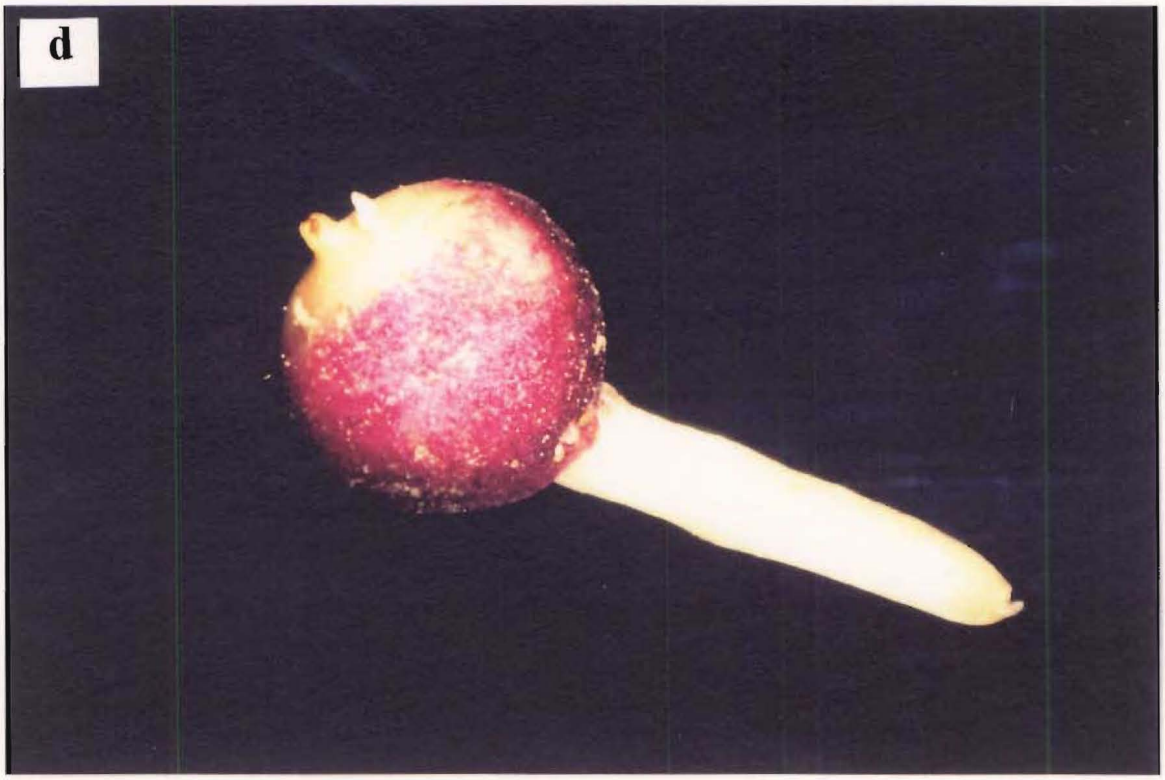
Figure 4.9 Development of anthocyanin pigmentation in Urenika tubers a) <1mm diameter b) ~1mm diameter c) ~1.5mm diameter d) ~2.5mm diameter e) 6mm diameter.

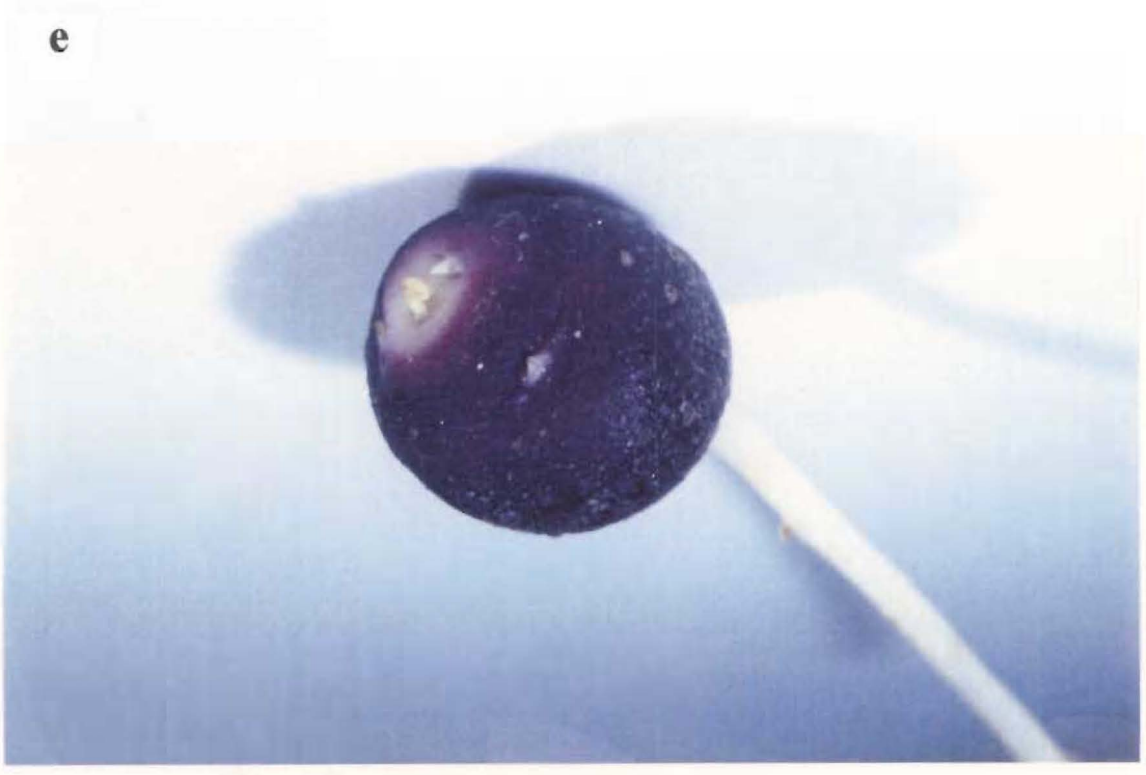
continued...

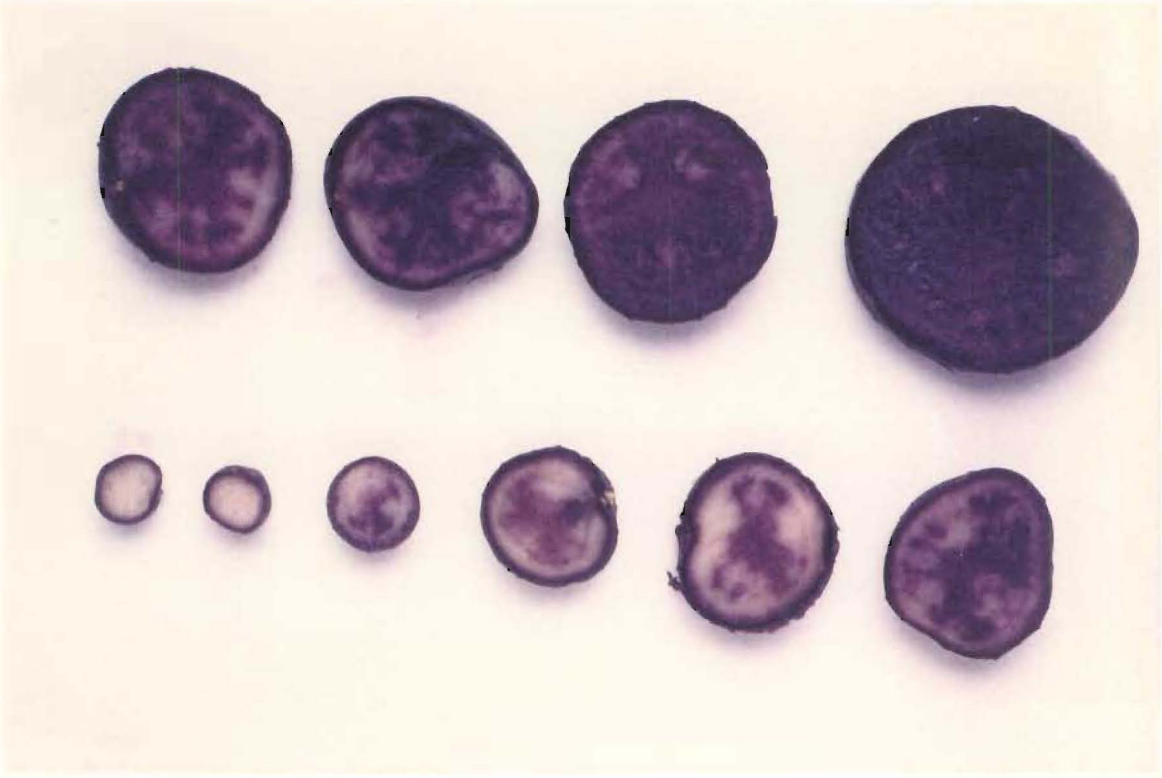
Figure 4.9 continued...

continued...

Figure 4.9 continued...







_____ = 10mm

Figure 4.10 Changes in anthocyanin pigmentation with increasing tuber size in Urenika flesh.

Consistent with the 1992/93 season, similar sized tubers had similar anthocyanin concentrations (for each cultivar), and there was no correlation between the age of the plant (date of harvest) and anthocyanin concentration. Therefore, data from similar sized tubers were combined and the results were analysed as before (Section 4.4.1.2). There was no anthocyanin present in the skin of Desirée tubers under 0.1g (Figure 4.11). As the tubers increased in size the anthocyanin concentration in the skin of the stem end of the tubers increased, followed later by an increase in anthocyanin concentration in the skin of the bud end of larger tubers. The stem end of tubers contained higher concentrations of anthocyanin than the bud end, until the tuber reached 150-200g, when the concentrations were similarly distributed (Figure 4.11). The concentration of anthocyanins in the skin of Desirée tubers increased with increasing tuber weight, until a maximum was reached when tubers were between 100 and 150g. This was similar to the pattern found in the 1992/93 season, except that in the 1993/94 season the tubers were slightly smaller because of less favourable weather conditions during the growth season.

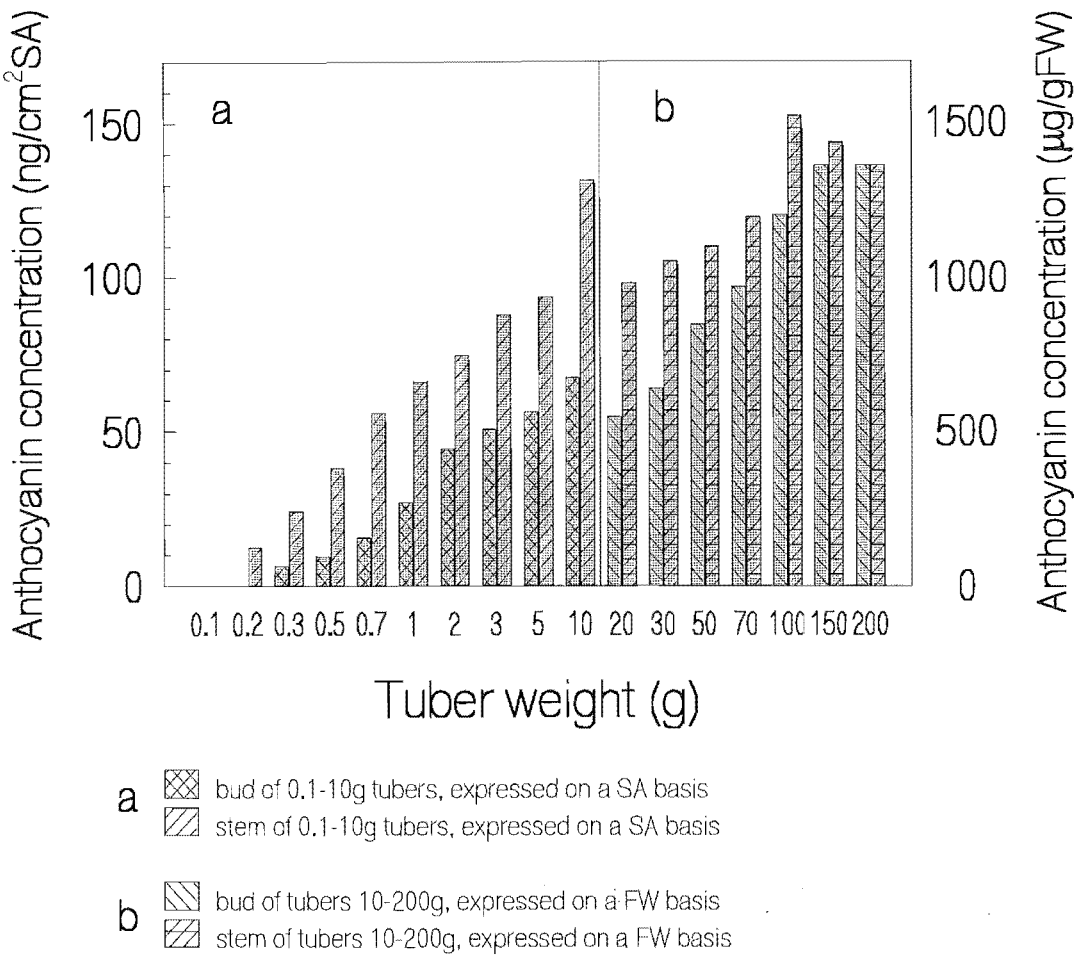


Figure 4.11 Anthocyanin concentrations in Desirée skin samples, separated into bud and stem ends of the tuber, averaged over the 1993/94 season. a) whole tuber extracted and anthocyanin concentration calculated per cm^2 of surface area (SA) and, b) samples of peeled skin only, and anthocyanin concentration calculated per gFW.

As for the 1992/93 season, the concentration of other flavonoids in Desirée tuber skin samples also increased with increasing tuber weight (from about 50µg/gFW to 400 µg/gFW), but although concentrations were slightly higher in the stem end of the tuber, there was no significant difference in flavonoid concentration between the stem and bud end (not shown). As before, phenolic acid concentration also increased (from about

1500μg/gFW to 3000μg/gFW) with increasing weight in smaller tubers (0-50g), but reached a maximum at about 50g (of 3000μg/gFW), and often decreased in larger tubers (not shown). The difference between the phenolic acids in the bud and stem ends showed the opposite pattern to the anthocyanins, with higher concentrations of phenolic acids found in the bud end than the stem end, until the tuber reached about 20g, after which there was no significant differences in phenolic acid concentration between the two ends of the tuber (not shown).

4.4.2 Levels of carotenoids in developing tubers

The carotenoid concentrations were measured in the skin and flesh of Arran Victory, Desirée, Red Flesh and Urenika cultivars throughout the 1992/93 season. The concentration of carotenoids was higher in the skin than the flesh of all tubers (Table 4.4). A typical example of the average change in carotenoids throughout development was shown by the skin and flesh of Desirée tubers (Figure 4.12). In all cultivars studied the carotenoid concentration decreased with increasing tuber weight, although a slight increase in concentration was found in the largest weight class of tubers for Arran Victory, Desirée and Red Flesh, but not for Urenika.

Table 4.4 Minimum and maximum carotenoid concentrations in tubers throughout development.

Cultivar	Tissue	Carotenoid concentration (ng/gFW)	
		minimum	maximum
Arran Victory	skin	506	1548
	flesh	293	744
Desirée	skin	440	1219
	flesh	368	1010
Red Flesh	skin	356	1914
	flesh	209	846
Urenika	skin	616	923
	flesh	355	487

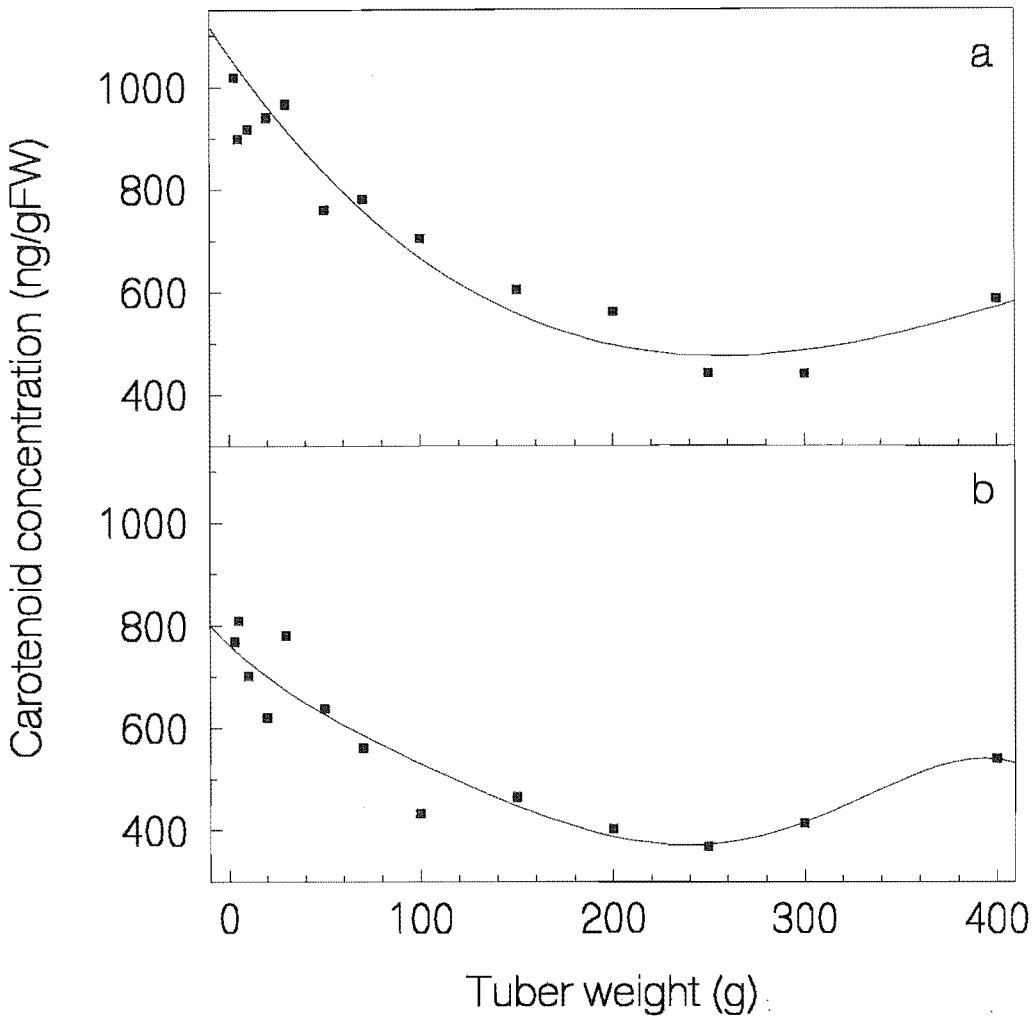


Figure 4.12 Changes in average carotenoid concentration in a) skin and b) flesh of Desirée tubers during development.

4.4.3 Effect of storage on the level of anthocyanins

4.4.3.1 1992/93 Season

Tubers of Arran Victory, Desirée, Red Flesh and Urenika were stored at 4°C, and sampled at intervals. The concentrations of anthocyanin increased in the skin of Desirée tubers (Figure 4.13a) and Arran Victory (not shown), and in the skin and flesh of Urenika tubers (Figure 4.13b and c), however there was no significant change in the anthocyanin concentrations in the skin or flesh of Red Flesh tubers (not shown) during storage. Additionally, the concentrations of flavonoids and phenolic acids were measured for Desirée tuber skin. The concentrations of flavonoids showed a similar

increase to the anthocyanins (flavonoid concentrations increased from about 350 to 430 μg/gFW, not shown), whereas the phenolic acid concentration increased from harvest to 120 days of storage (increased from about 2500 to 2800μg/gFW), after which there was no change (not shown). All individual compounds within the anthocyanin, flavonoid or phenolic acid classes showed similar proportional increases, and there was no change in the proportion of individual compounds within these classes during storage.

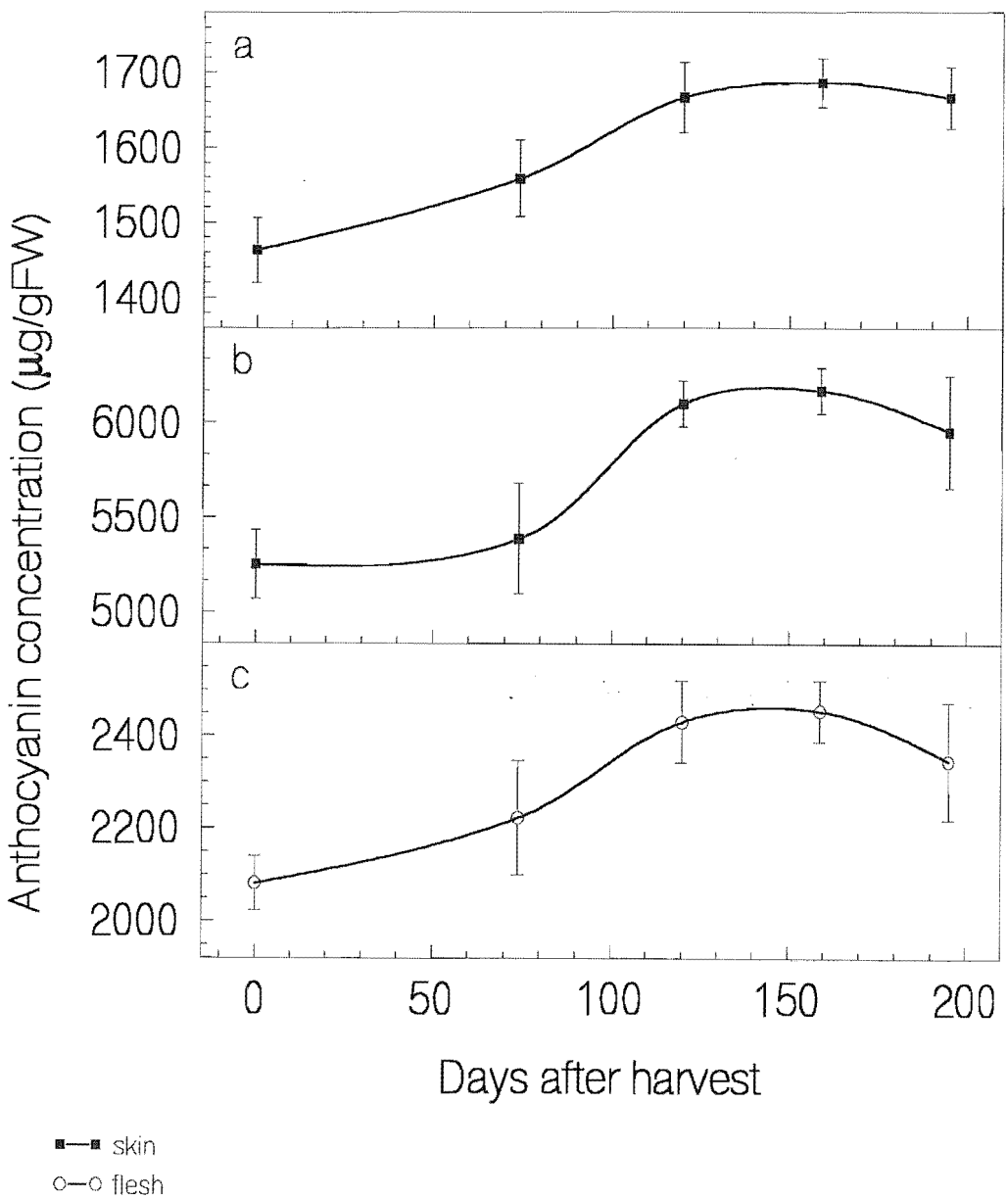


Figure 4.13 Changes in anthocyanin concentration in a) Desirée skin, b) Urenika skin and c) Urenika flesh of tubers during storage at 4°C for the 1992/93 season.
Error bars represent ± 1 standard error.

4.4.3.2 1993/94 Season

Following the analysis of cold stored tubers from the second season, the anthocyanin concentrations again showed increased concentrations in the skin of Desirée tubers (Figure 4.14a) and Arran Victory tubers (not shown), and in the skin and flesh of Urenika tubers (Figure 4.14b and c) during storage, whilst no change was shown by the skin and flesh of Red Flesh tubers (not shown).

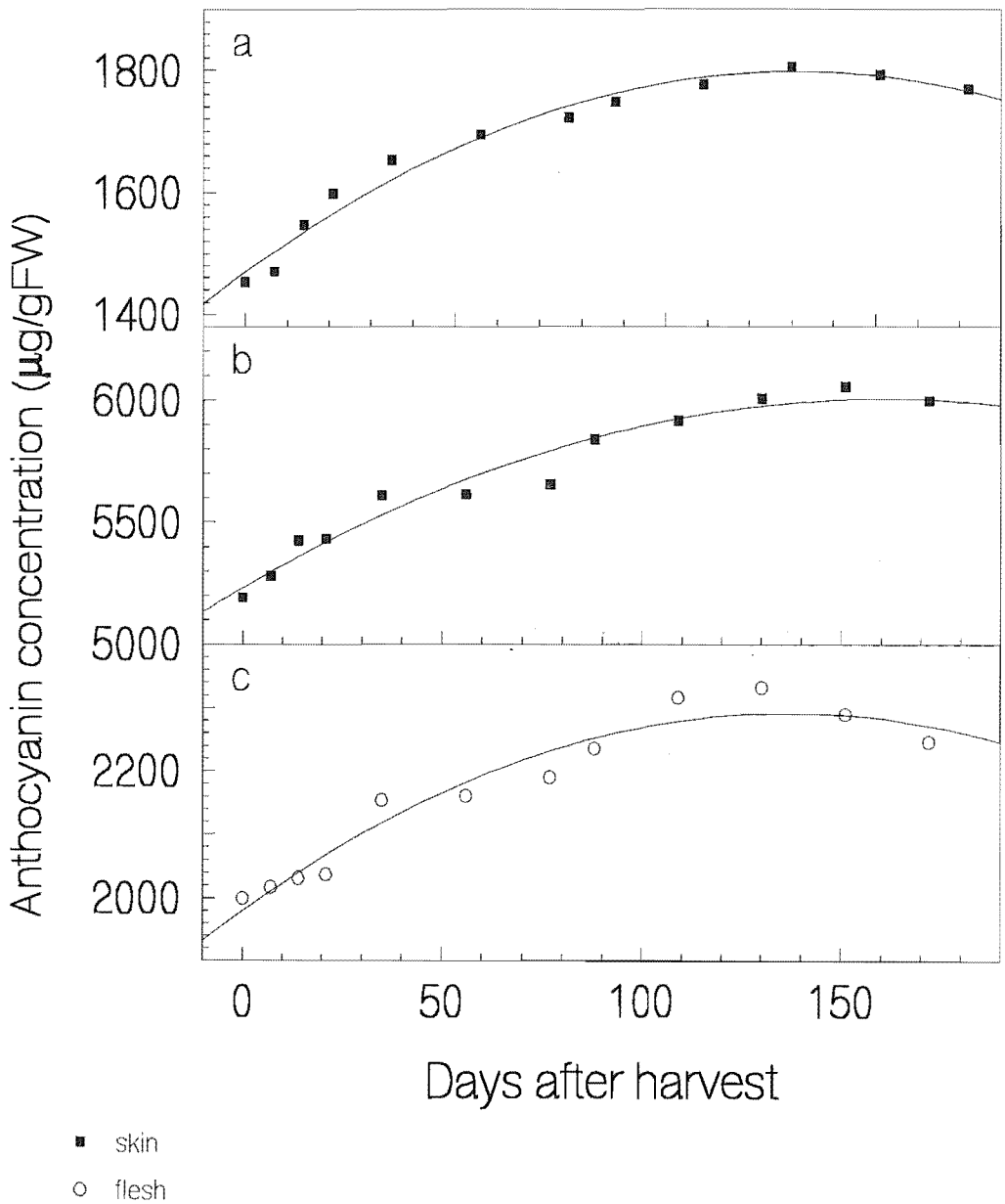


Figure 4.14 Changes in anthocyanin concentration in a) Desirée skin, b) Urenika skin and c) Urenika flesh of tubers during storage at 4°C for the 1993/94 season.

For the 1992/93 season it was uncertain whether or not the increase in anthocyanins measured during storage was because of the biosynthesis of anthocyanins, or the loss of water with an accompanied increase in %DM of the tubers, because the anthocyanin concentrations were expressed on a fresh weight basis. Therefore, the fresh weight and %DM of each tuber was measured throughout the 1993/94 storage period. Although up to 5% of the initial fresh weight had been lost after 170 days of storage, and an additional 2% lost in sprout growth (shown by Desirée tubers in Figure 4.15), there was no change in the %DM (not shown). Therefore, the pattern of change in anthocyanin concentrations when calculated on a dry matter basis (not shown) was very similar to those shown in Figure 4.14 (where results are presented on a FW basis). The changes in FW and %DM were similar for the other cultivars.

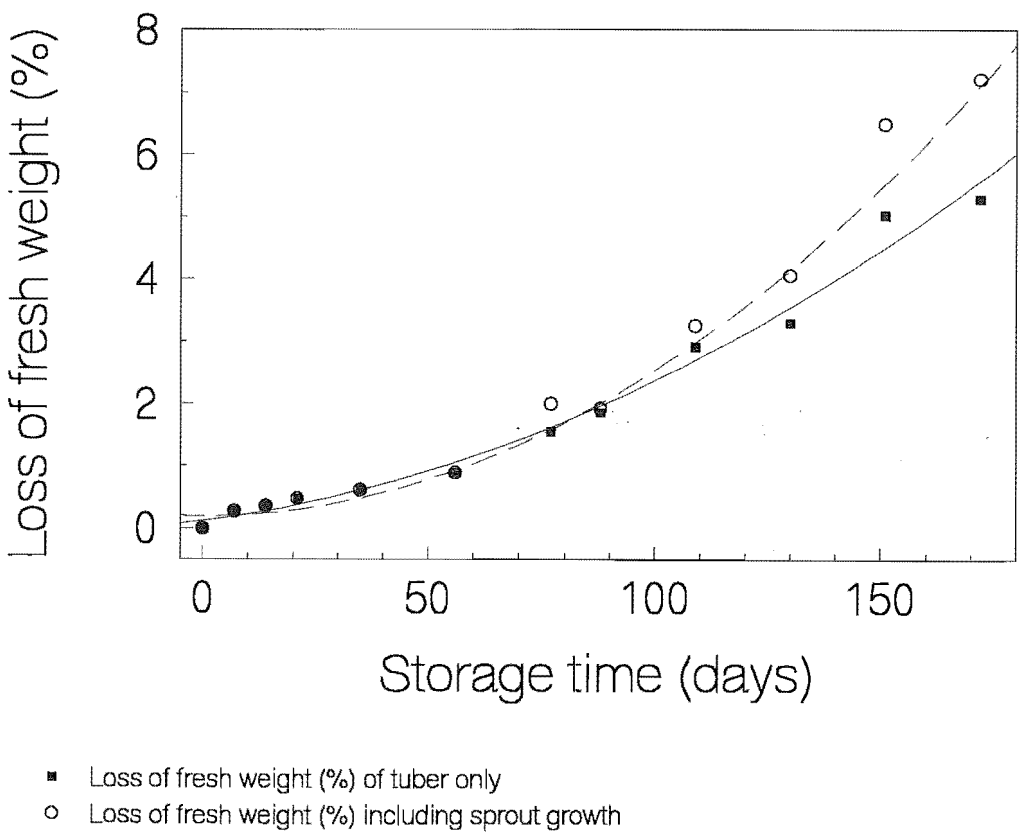


Figure 4.15 Percentage loss of fresh weight from Desirée tubers during storage at 4°C for the 1993/94 season.

4.4.3.3 Effect of storage temperature on anthocyanin concentration

Tubers were stored at four different temperatures and sampled fortnightly for analysis of anthocyanin concentration. The anthocyanin concentration of tubers stored at 4°C increased gradually as before (Sections 4.4.3.1 and 4.4.3.2), whereas in tubers stored at higher temperatures, the anthocyanin concentration gradually decreased with increasing temperature. Figure 4.16 shows the anthocyanin concentrations for Desirée tubers after ten weeks of storage at each of these temperatures. The average loss of fresh weight from five tubers at each temperature is shown in Figure 4.17, where tubers stored at higher temperatures suffered from larger losses of fresh weight. The tubers stored at 10°, 18° and 26°C also sprouted to a considerable extent (9.1, 8.3 and 5.0% of the original fresh weight respectively was made up of sprout growth), whilst tubers stored at 4°C showed no sprout growth after ten weeks. Tubers at these three higher temperatures became wrinkled and increasingly difficult to peel and obtain flesh-free skin samples. There was no significant change in %DM for tubers stored at any of the four temperatures over the ten week storage period.

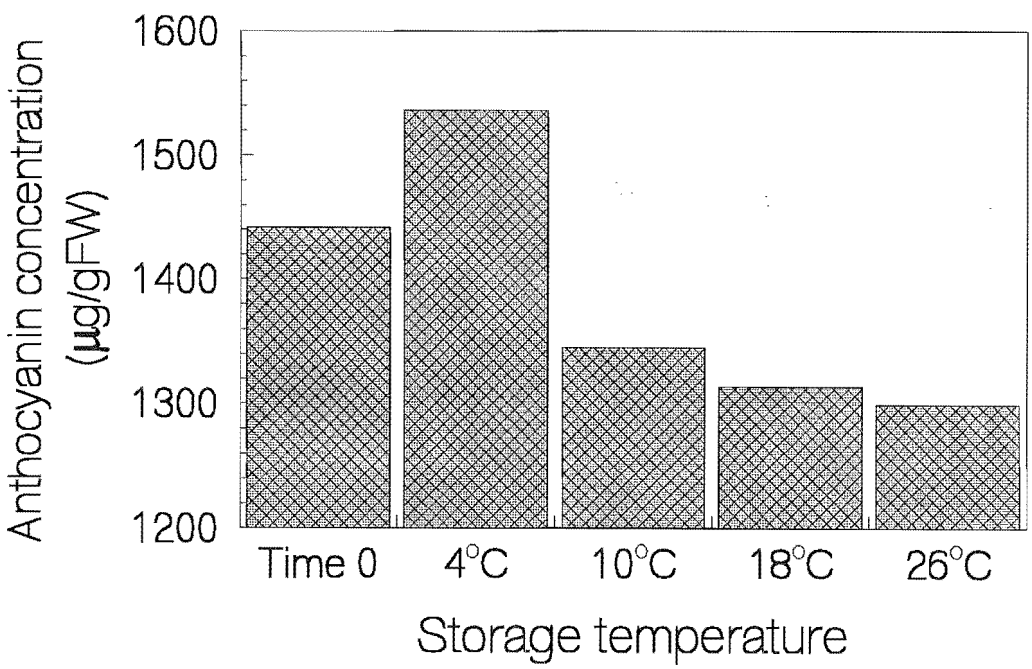


Figure 4.16 Change in anthocyanin concentration of Desirée tubers after ten weeks of storage at a range of temperatures.

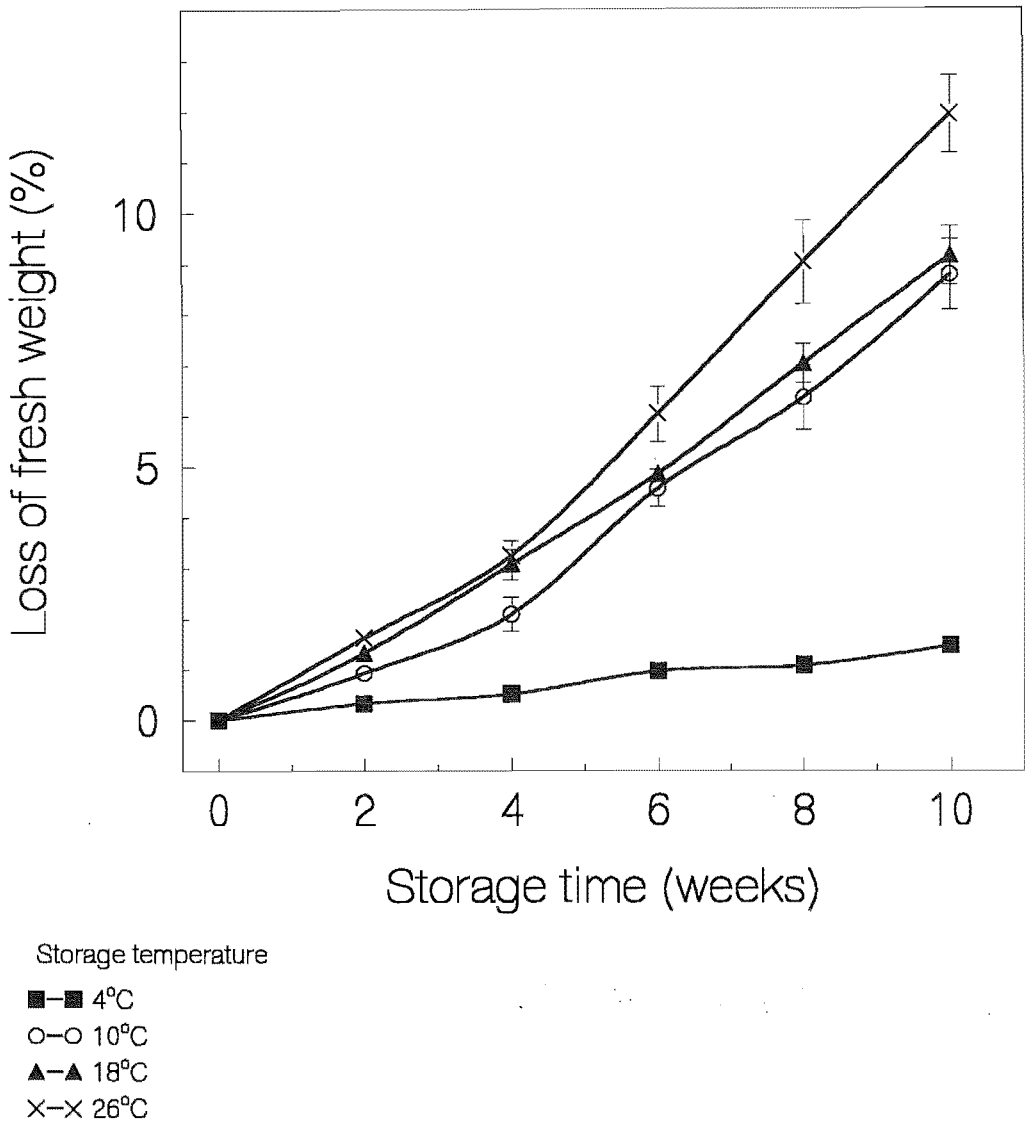


Figure 4.17 Loss of fresh weight of Desirée tubers after ten weeks storage at a range of temperatures. Error bars represent ± 1 standard error.

4.4.4 Distribution of anthocyanin in tubers

As reported earlier (Section 4.4.1.2), the anthocyanin concentration in the stem end of tubers was higher than in the bud end throughout most of the tuber development. Anthocyanin concentration was measured in the skin of five approximately equal slices of Desirée tubers (50-70g and 70-100g tubers) while the plants were still actively growing, and again after storage for four months at 4°C. In actively growing tubers there was a

gradient of anthocyanin from high concentrations at the stem end to lower concentrations at the bud end, and this difference was more pronounced in the smaller (50-70g) tubers (Figure 4.18a) (where anthocyanin concentration at the bud end was 75% of that at the stem end), than the larger (70-100g) tubers (Figure 4.18b) (where anthocyanin concentration at the bud end was 88% of that at the stem end). After four months of cold storage the distribution of anthocyanin was reversed, with much higher concentrations in the bud end than the stem end (Figure 4.18c).

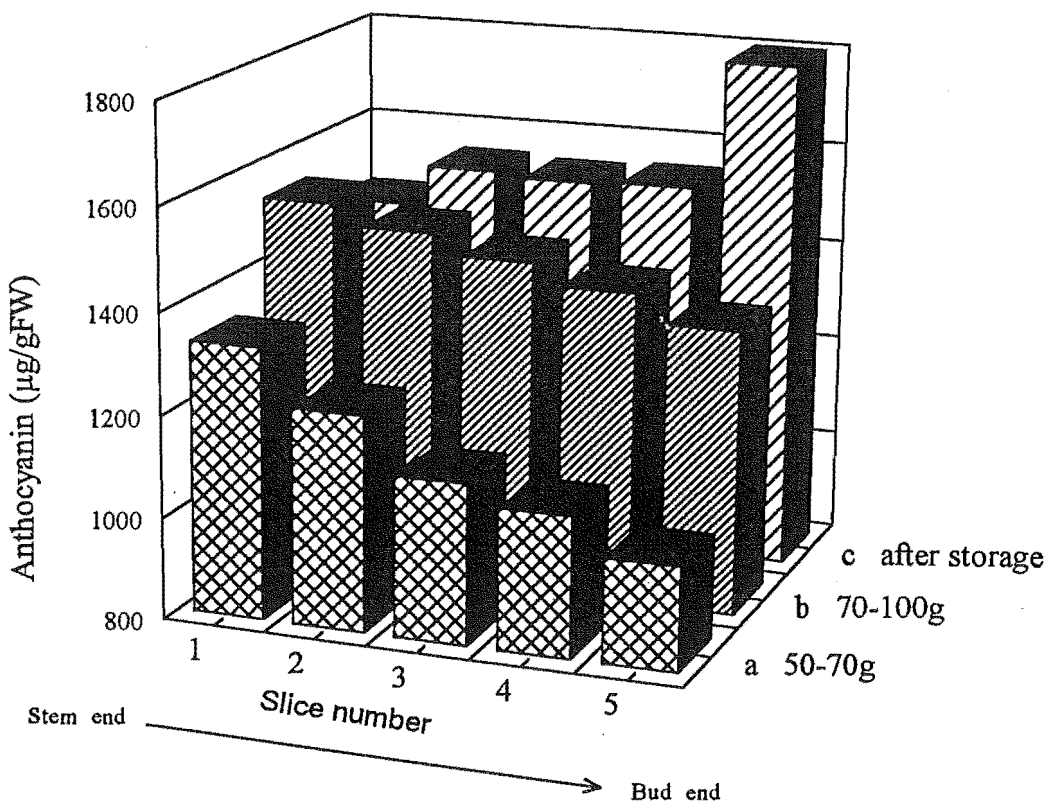


Figure 4.18 Distribution of anthocyanin concentration in the skin of Desirée tubers a) 50-70g and b) 70-100g during development, and c) after four months cold storage.

The concentration of anthocyanin was also measured in skin and flesh of Arran Victory, Red Flesh and Urenika tubers after storage at 4 C. Urenika also showed a higher anthocyanin concentration towards the bud end in both the skin and flesh (Figure 4.19a and b). Arran Victory showed low anthocyanin concentration in the middle of the tuber, with medium concentrations at the stem end and high concentrations at the bud end, in both skin and flesh samples (Figure 4.19c and d). Anthocyanin concentrations in the skin

and flesh of Red Flesh tubers (not shown) showed a similar pattern to that of Arran Victory skin (Figure 4.19c). As well as the extraction and measurement of the anthocyanin concentration in slices of stored tubers, the colour density of Urenika flesh slices was measured by image analysis (Figure 4.20). Thus, showed a similar pattern to that of the anthocyanin concentration in Urenika flesh determined by extraction and spectrophotometric measurement (Figure 4.19a).

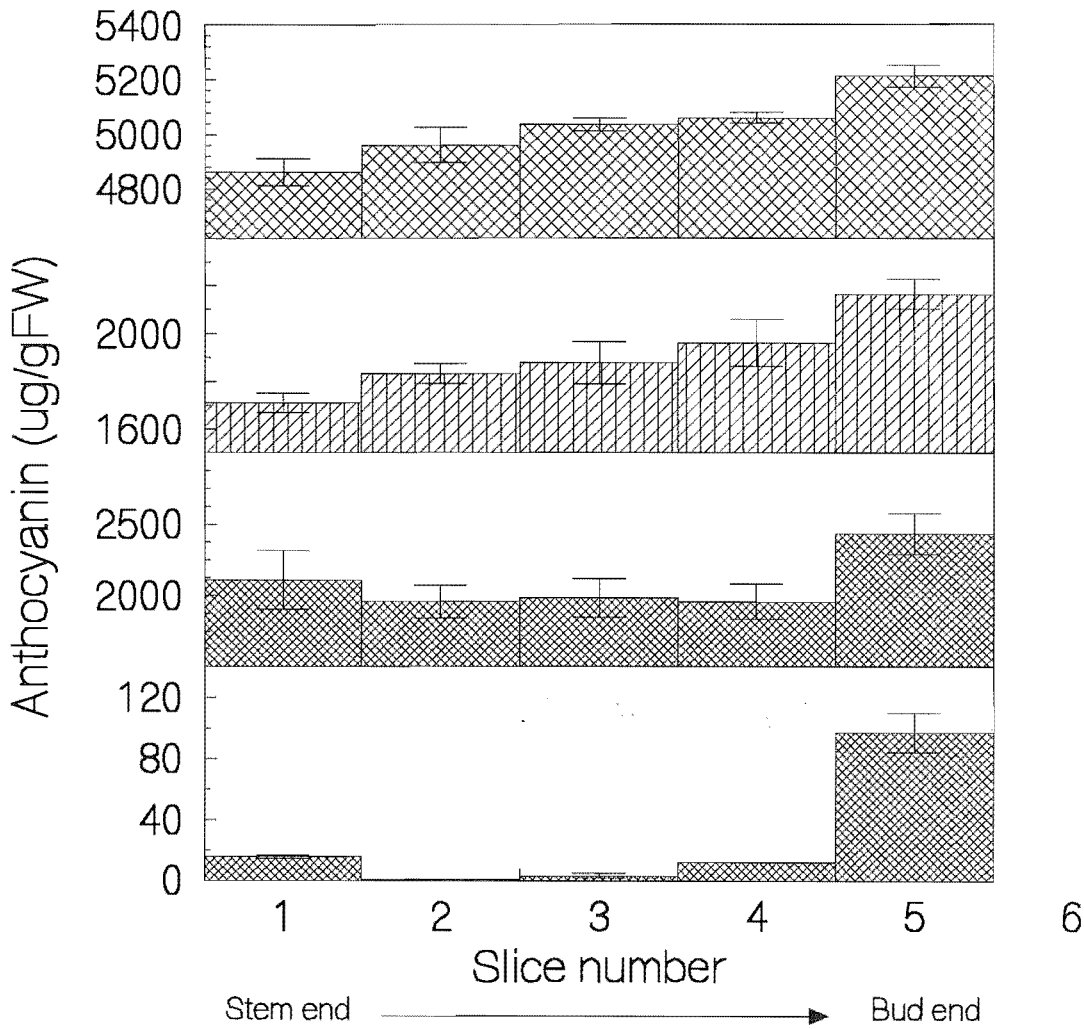


Figure 4.19 Distribution of anthocyanin concentration in tubers after four months of storage at 4°C. a) Urenika skin b) Urenika flesh c) Arran Victory skin and d) Arran Victory flesh. Error bars represent ± 1 standard error.

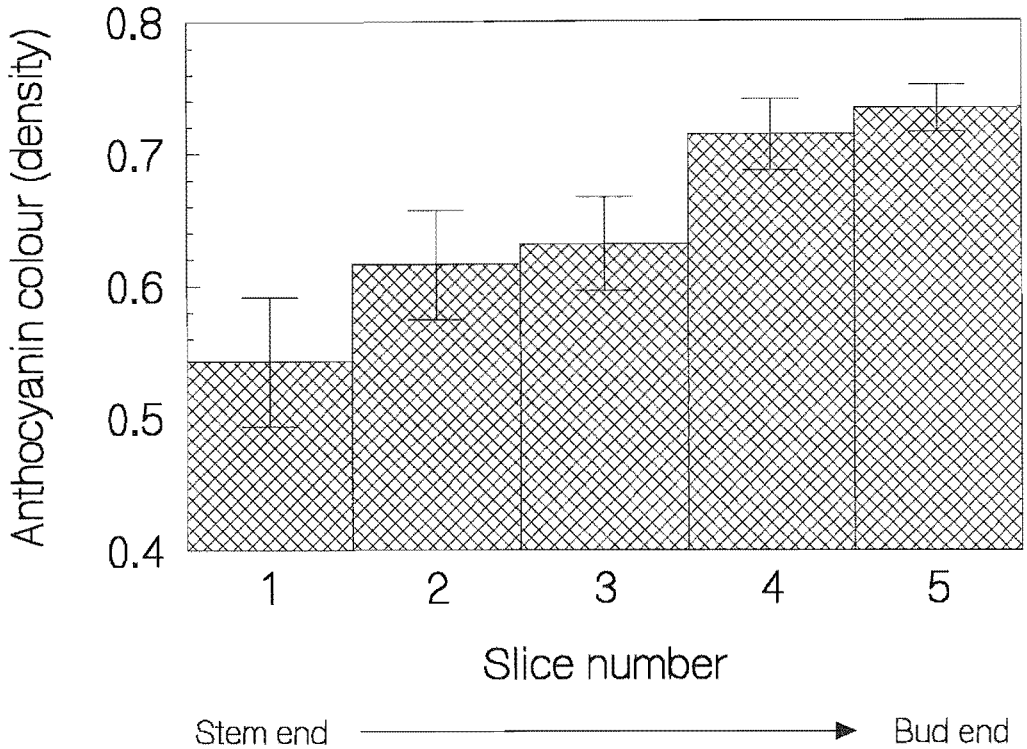


Figure 4.20 Distribution of anthocyanin pigment in the flesh of Urenika tubers after four months of storage at 4°C, as measured by image analysis. Error bars represent ± 1 standard error.

4.5 Discussion

4.5.1 Anthocyanin metabolism

The newly initiated tubers contained little or no anthocyanin. The size of the tuber when anthocyanin pigmentation was first observed appeared to depend on the cultivar. In the cultivars with strongly coloured tubers, such as Red Flesh and Urenika, production occurred immediately after tuber initiation with maximum anthocyanin concentrations in the skin reached before the tuber weighed about 5g. However, in less strongly coloured cultivars, such as Arran Victory and Desirée, maximum colour was not reached until the tuber weighed about 200g. In small tubers with no colour (e.g. Desirée), anthocyanin was found first at the stem end of the tuber, with the pigment gradually becoming visible further towards the bud end, with the bud end sometimes remaining white until tubers weighed over 50g. Therefore, the biosynthesis, or accumulation of anthocyanin occurred

first in the stem end, and then the anthocyanins either 1) migrated towards the bud end, or more likely, 2) were then synthesised in a gradual manner towards the bud end. The anthocyanins were unlikely to be transported during the development of the tuber because they are relatively large molecules, and are stored in the vacuole, and it is more likely that the smaller precursor molecules were transported. In Desirée tubers, the stem end contained higher concentrations of anthocyanin than the bud end throughout the period of tuber growth. It was only when the tubers had stopped increasing in size that the anthocyanin concentrations in the stem and bud ends became similar. This suggests the presence of some compound which is transported via the rhizome to the developing tuber, but whether this is a hormone or some other signal, or simply the flavonoid precursors is still unclear. It is proposed that this compound(s) reached the stem end first, where anthocyanins were produced and the compound(s) was gradually transported to the bud end, where anthocyanin biosynthesis followed at a later stage.

After cold storage, the distribution pattern of anthocyanins was reversed from that found in developing tubers, with the highest concentration found in the bud end. It was thought that after cold storage, when the tuber begins to sprout, the starch was converted to sugars which were mobilised towards the bud end (sprouts) and used for growth. At this time, after cold storage, the tubers had higher concentrations of anthocyanin in the bud end, and because the concentration of anthocyanin increased during cold storage, it was proposed that the increase in sugars (a substrate for anthocyanin biosynthesis - refer to Section 5.1.1) promoted the anthocyanin biosynthesis. It was likely that because the sugars were mobilised towards the bud end (and the sprouts), the anthocyanin biosynthesis was higher in the bud end than in the stem end, where the concentration of sugars was lower. However, a lot more work is required to investigate more fully the distribution and changes of anthocyanin, sugar, and other flavonoid precursor concentrations during tuber development and cold storage to substantiate these hypotheses. These further investigations were outside the scope of this thesis.

In the cultivar Urenika, which has purple/black skin and flesh in mature tubers, the production of anthocyanin in the skin occurred almost immediately after tuber initiation however, the flesh remained completely white until tubers reached about 5g (5-10mm in diameter). The production of anthocyanin in the flesh then began from the stem end, and gradually increased with the bud end producing anthocyanin last. Therefore, in the more strongly coloured tubers (e.g. Urenika) the anthocyanin concentration reached a maximum in much smaller tubers, than in weaker coloured tubers (e.g. Desirée). However, in all cultivars studied, the biosynthesis of anthocyanins followed the same

pattern of appearance, being formed in the stem end first, followed by formation in the bud end.

When the anthocyanin, flavonoid and phenolic acid concentrations were measured throughout the development of the tubers, no correlation was found between these concentrations and the harvest date (*i.e.* age of the plant). Although the average tuber size (weight) for each plant harvested generally increased throughout the season, this was not always the case because initiation of new tubers took place over about a two month period, so a range of tuber sizes were present at each harvest. Tuber size also depended to a lesser extent on the number of tubers present on individual plants and environmental conditions. However, the concentrations of anthocyanins, flavonoids and phenolic acids were correlated with the size of the tuber (not the sampling date or age of the plant). Because of variation between plants, it would be useful to carry out further experiments with more plants and a larger sample of tubers within each size class (more than the six to eight tubers in each class that were used for this study). There would be no need for the analysis of tubers throughout the season as long as the range of tuber sizes were sampled, because the age of the plant *per se* had no effect on the concentration of the phenolics.

Within each plant, the concentration of anthocyanins, flavonoids and phenolic acids per gFW increased with increasing tuber weight. Phenolic acid concentrations reached a maximum in tubers of about 100g, whilst anthocyanin and flavonoid concentrations reached a maximum slightly later in tubers of about 150g, before decreasing slightly. The concentration of phenolic acids reached a maximum before that of the anthocyanins and flavonoids, possibly because they are earlier intermediates in the phenolic and flavonoid pathways. The slight decrease in concentration of these phenolics compounds in large tubers probably results from concentrations being measured per gFW. It is thought that the synthesis of phenolic compounds had slowed down or stopped, and because the tuber was still increasing in size this resulted in a reduced concentration per gFW (dilution), rather than the degradation or metabolism of these compounds.

The cold storage of tubers caused a gradual, but significant increase in anthocyanin concentration over a period of five months. It was initially thought that this increase may have been caused by the loss of water and associated increase in %DM, rather than the synthesis of anthocyanin. However, in the second season when this experiment was repeated and the %DM measured, there was a decrease in FW of the tubers, although no change in %DM. This suggests that the increase in coloured pigments during storage was because of the synthesis of anthocyanin molecules. There may also have been a change in colour owing to a change in the vacuolar medium, such as in pH or

copigments, but neither of these effects would have been detected with the spectrophotometric method that was used. The pH of samples was standardised before measurement and the samples were redissolved in methanol, so no copigmentation would have occurred (because copigmentation can occur only in aqueous solutions).

Before the experiments on the effect of storage temperature were carried out, the tubers were stored outside for six weeks (at 0-10°C). During this time the anthocyanin concentration increased from 460μg/gFW (Figure 4.14a) to 640μg/gFW (Figure 4.16). During the experimental storage period of ten weeks at 4°C the anthocyanin concentration increased further, however tubers stored at 10°, 18° and 26°C showed a decrease in anthocyanin concentration. Even so, the anthocyanin concentration of the tubers stored at these higher temperatures only reduced to 500μg/gFW, which was still slightly higher than the concentration at the harvest date (460μg/gFW). Therefore, it is not known if this decrease at the three higher temperatures would have occurred without the initial six week storage period. It would have been interesting to repeat this experiment and store the tubers at different temperatures immediately after harvest to see if a decrease still occurred at higher temperatures, but time constraints did not permit this to be carried out.

Cold storage of tubers is known to cause the production of sugars from starch (cold sweetening) (Burton, 1978). Because sugars are one of the precursors of anthocyanins (Section 5.1.1), this increase in sugars may have played a part in the synthesis of anthocyanins during cold storage, especially since only tubers in cold storage showed an increase in anthocyanin concentration. A number of workers have reported that plants produce anthocyanins under high sugar conditions (reviewed in Hrazdina, 1982), and Harborne (1967) proposed that the excess sugar in the cells upsets metabolism in the direction favouring anthocyanin synthesis.

Sprouting occurred in tubers stored at the three higher temperatures (10°, 18° and 26°C) and the decrease in anthocyanin concentration was related to the amount of sprouting, in that there was a larger decrease in anthocyanin concentration in tubers with a larger weight of sprouts. The sprouts also showed anthocyanin colouration (in dark stored tubers), so it is thought that the anthocyanin was either 1) transported from the tuber to the sprouts or, more likely 2) degraded in the tubers to be re-synthesised in the sprouts. Sprouting has also been associated with the metabolism of sugars, but in this case the sugars were transported to the growing sprouts. It is suggested that the decreased anthocyanin concentration in stored tubers is associated with the sprouting, especially as in the tubers stored at 4°C the decrease in anthocyanin concentration in the last month of storage (Figures 4.13, 4.14 and 4.15) coincided with the sprouting of these tubers. It is

thought that the increased anthocyanin concentration in cold stored tubers (4°C) was because of an increase in sugar concentration in the tubers. These storage experiments showed that both a net synthesis and degradation of anthocyanins can occur in potato tubers, which suggests that there may be continual metabolism of anthocyanins as tubers respond to environmental conditions; analogous to that which occurs with starch/sugar metabolism. It would be of great interest to pursue further this line of research into the effect of different storage temperatures on the metabolism of anthocyanins and flavonoids, to determine what effects the commercial storage of tubers has on the colour of tubers, and to elucidate further the effect of sugars, cold sweetening and sprouting in tubers on the anthocyanin concentration.

Most tubers are stored before private or commercial use. Therefore, in tubers where colour is important, care needs to be taken to ensure that storage conditions are optimum for maximum tuber colour, because the anthocyanin concentration changes response to the storage conditions (e.g. storage temperature, length of storage time, etc.).

4.5.2 Carotenoid metabolism

The measurement of carotenoids throughout the development of tubers was carried out as a minor project because carotenoids are the other main pigment class in potatoes apart from anthocyanins. It would have been more appropriate to calculate the carotenoid concentration from a calibration curve of violaxanthin and/or lutein instead of β-carotene, because potatoes have been found to contain mostly the former, and none or extremely minor amounts of β-carotene, however only β-carotene was readily available for use as a standard. However, the measurement of carotenoids was carried out only to give an indication of the relative levels present and the changes occurring in the five cultivars, and to compare this with the anthocyanins, therefore the changes rather than absolute values of carotenoids were important. It was interesting to discover that the concentrations of carotenoids followed a completely opposite pattern to that of anthocyanins, flavonoids and phenolic acids during the growth of tubers, with carotenoid concentration decreasing with increased tuber weight. The concentration of carotenoids in mature tubers was low in most cultivars and, when anthocyanin pigmentation was also present, the carotenoids did not appear to contribute towards the final colour of the tuber, except in some flesh samples. The concentration of carotenoids was higher in the skin than the flesh in all cultivars.

4.5.3 Conclusions

The major conclusions and answers to the questions raised in the aims of this chapter, are summarised below.

1) The biosynthesis of anthocyanins coincided with tuber initiation in the cultivars with intensely coloured mature tubers (*i.e.* high concentrations of anthocyanin), and after a small increase as the smaller tubers increased in size, anthocyanin concentrations remained relatively constant (although because tubers were increasing in weight, anthocyanin was still being synthesised to maintain a constant concentration). However, in cultivars with weakly coloured mature tubers (*i.e.* lower anthocyanin concentrations), the developing tubers remained white for longer, and concentrations increased gradually with increasing weight, up to a maximum concentration at a certain tuber weight depending on the cultivar, before decreasing slightly. In Desirée tubers, the concentration of flavonoids followed a similar pattern to the anthocyanins, whilst the phenolic acids reached a maximum concentration at a slightly lower tuber weight than the anthocyanin and flavonoids.

2) The distribution of anthocyanins altered during tuber development and also during cold storage. In very small developing tubers the anthocyanins were found first in the stem end of the tuber, whilst the bud end remained white. As the anthocyanin concentration increased during tuber development, the concentration increased over the whole tuber, and the concentration at the stem end was always higher than that at the bud end, until the tuber reached maturity when the concentration was approximately equal in both ends.

3) During cold storage the anthocyanin concentration in the tubers increased. After cold storage tubers had a higher concentration of anthocyanin in the bud end than the stem end - the opposite pattern to that found in developing tubers. The storage of tubers at higher temperatures did not show this increase in anthocyanin concentration. The increased colour in cold stored tubers was discussed in terms of its relationship "cold sweetening" and the increased concentration of sugars in these tubers.

4) The concentration of carotenoids decreased during tuber development, showing the opposite pattern to the increased anthocyanin concentrations. The carotenoids did not appear to have any effect on the visible colour of mature uncut "coloured" tubers (*i.e.* tubers containing anthocyanin in the skin), because the anthocyanin colour was more intense than the carotenoid colour. However, when anthocyanins were not present, the carotenoids provided the majority of the colour, especially in the flesh.

CHAPTER 5

Biosynthesis and regulation of the anthocyanins and other flavonoids

5.1 Introduction

5.1.1 Flavonoid biosynthesis

Two main types of metabolism are thought to exist in plants, primary and secondary metabolism. Primary metabolism is the metabolism of compounds which are absolutely necessary for the normal functioning and growth of the plant, and includes the metabolism of carbohydrates, proteins, and nucleic acids. Secondary metabolism is the metabolism of compounds which are not obviously vital to the plants life, and includes the synthesis of compounds such as phenolic acids, flavonoids, terpenoids, alkaloids, etc., (Neumann *et al.*, 1985).

The results of feeding experiments using ^{14}C-labelled precursors have indicated that the fifteen carbon skeleton of flavonoids is derived from two separate pathways involved in primary metabolism - the malonate (including the acetate) pathway and the shikimic acid pathway (Figure 5.1). The A-ring of flavonoids (Figure 1.2) is formed by the head to tail condensation of one acetyl CoA and two malonyl CoA units; whilst the B- and C-rings are derived from 4-coumaroyl CoA, a C_6-C_3 phenylpropanoid (Stafford, 1990). The synthesis of these precursors is discussed below.

5.1.1.1 Precursor pathways

The flavonoid precursors are derived from carbohydrates (Figure 5.1). Malonyl CoA is produced via the malonate pathway, and the 4-coumaroyl CoA precursor via the shikimic acid and phenylpropanoid pathways.

Note that throughout this chapter light intensity is expressed in Watts/m^2, rather than μmol/m^2/sec or μEinstein/m^2/sec which would have been more correct in most cases. Watts/m^2 was used because in Figure 5.16 it was necessary to measure actual light intensity at each wavelength, rather than photosynthetically active light, and therefore, for consistency Watts/m^2 was used in all cases.

A) Malonate pathway

Acetyl CoA is formed directly from carbohydrates as an integral part of the cell's primary metabolism. Malonyl CoA is formed in a complex reaction from acetyl CoA and CO_2, catalysed by acetyl CoA carboxylase (Figure 5.1), in the presence of ATP and Mg^{2+}. Malonyl CoA is a precursor for flavonoid biosynthesis and supplies the C_2 units used in the synthesis of the flavonoid A-ring. Malonyl CoA also has a number of other uses in the cell; for example, it supplies the C_2 units used for the synthesis of cuticular waxes, stilbenoids, anthroquinones and malonyl amino cyclopropane-1-carboxylic acid. It is thought that acetyl CoA carboxylase exists as a series of isozymes in different cellular compartments to participate in such a varied series of reactions (Stafford, 1990).

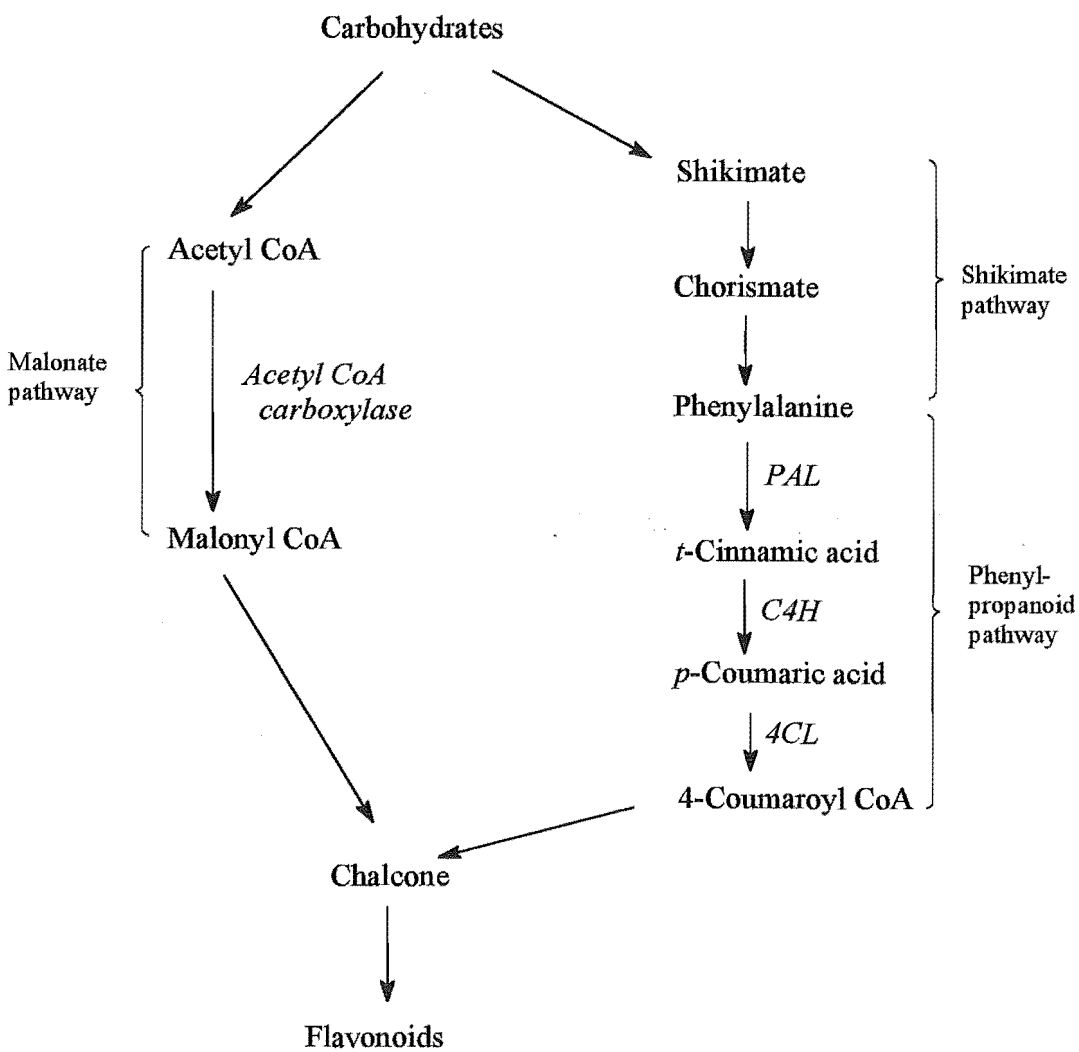


Figure 5.1 Precursor pathways of flavonoid biosynthesis: the production of precursors from carbohydrates via the acetate, shikimic acid and phenylpropanoid pathways (PAL = phenylalanine ammonia-lyase, C4H = cinnamic acid 4-hydroxylase, 4CL = 4-coumarate:CoA ligase).

B) Shikimic acid pathway

The shikimic acid pathway produces phenylalanine from carbohydrates via the production of chorismate, and is the main route to the aromatic (phenolic) amino acids, phenylalanine and tyrosine, in higher plants (Jensen, 1985). Although phenylalanine is the main substrate for the phenylpropanoid pathway leading to the production of 4-coumaroyl CoA, tyrosine may also be used in a parallel reaction leading into the phenylpropanoid pathway and production of 4-coumaroyl CoA.

C) Phenylpropanoid pathway

The phenylpropanoid pathway is involved in secondary metabolism, and forms the basic C_6 aromatic rings for all major plant phenolics, including the precursor used in the production of flavonoids. Other major plant phenolics produced via the phenylpropanoid pathway include the benzoic and cinnamic acids, which are important components in the synthesis of cells walls and lignin (Ribéreau-Gayon, 1972). The phenylpropanoid pathway involves three enzymes; phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL) (Figure 5.1). A brief description of these enzymes and their regulation is included below.

1) Phenylalanine ammonia-lyase (PAL)

PAL is often regarded as the key enzyme of the phenylpropanoid pathway, and in the biosynthesis of flavonoids and many other phenolics (Hahlbrock *et al.*, 1976; Stafford, 1990). It catalyses the deamination of L-phenylalanine to yield *t*-cinnamic acid and ammonia (Figure 5.2), which is the first committed step for phenylpropanoid biosynthesis in higher plants, and links primary and secondary metabolism.

PAL was first discovered in barley seeds by Koukol and Conn (1961), and since then has been studied extensively, and also been purified and characterised from a number of plant and fungal sources (Hanson and Havir, 1979). The purified PAL enzyme from potato tubers was found to have a molecular weight of 332 kDa (Havir and Hanson, 1968), and to be composed of four equal subunits, each with a molecular weight of 83 kDa (Havir and Hanson, 1973), which is similar to that found in many other plants (reviewed in Stafford, 1990). Wide variations in levels of PAL activity from plants have been recorded, and levels appear to depend on the genotype, age, developmental status, organ and even tissue of the plant. A number of studies have been carried out using developing seedlings, in which PAL activity changes dramatically during the first few days (Weissenböck, 1975; Knogge and Weissenböck, 1986).

Increased PAL activity has been shown to be associated with the synthesis of phenolics, including anthocyanins and other flavonoids (Hahlbrock *et al.*, 1971a and b; Cheng and

Breen, 1991). PAL activity, and therefore the synthesis of phenolics, is affected by a number of factors including light, wounding, pathogen attack, temperature, mineral and water nutrition, and growth regulators (Camm and Towers, 1977). A number of early studies found increased PAL activity after illumination of potato tuber disks (Zucker, 1965, 1968; Sacher *et al.*, 1972; Lamb and Rubery, 1975). Experiments using inhibitors of transcription and translation, and also radioactive feeding experiments, have suggested that this increased PAL activity after exposure to light is caused by the synthesis of PAL mRNA and *de novo* synthesis of the enzyme protein (Creasy and Zucker, 1974; Ishizuka *et al.*, 1991). Following this increase in PAL activity, the subsequent decrease in activity has been found to be caused by decreased PAL synthesis, coupled with PAL inactivation. This inactivation has been found to be caused by an inducible, probably proteinaceous, inhibitor of the PAL enzyme (Hanson and Havir, 1981).

A similar enzyme, tyrosine ammonia-lyase (TAL), which catalyses the deamination of tyrosine to produce *p*-coumaric acid, is also found to be associated with flavonoid biosynthesis in some cases. However, although TAL activity has been found in a number of plants, it has not been found in extracts from potatoes (reviewed in Stafford, 1974), and will not be considered here.

2) Cinnamic acid 4-hydroxylase (C4H)

C4H is a microsomal enzyme containing cytochrome P-450, and catalyses the O_2-dependent hydroxylation of *t*-cinnamic acid to form *p*-coumaric acid (Figure 5.2). Similarly to PAL, C4H activity has been reported to increase in response to light and wounding in some plants (Creasy and Zucker, 1974), and therefore the phenylpropanoid pathway and production of phenolics is very responsive to these environmental changes.

3) 4-coumarate:CoA ligase (4CL)

4CL catalyses the synthesis of the CoA ester, 4-coumaroyl CoA, from *p*-coumaric acid (Figure 5.2), and is dependent on ATP and Mg^{2+}.

4-Coumaroyl CoA, together with acetyl CoA and two molecules of malonyl CoA, are the substrates for the synthesis of the C_{15} flavonoid intermediate.

5.1.1.2 Specific flavonoid pathways

From the formation of the flavonoid precursors, and the formation of the C_{15} flavonoid intermediate, there are a number of specific flavonoid pathways which provide the biosynthetic routes to the various classes of flavonoids, such as flavonols,

dihydroflavonols, flavones, anthocyanins, etc. The enzymes involved in these reactions will be discussed briefly.

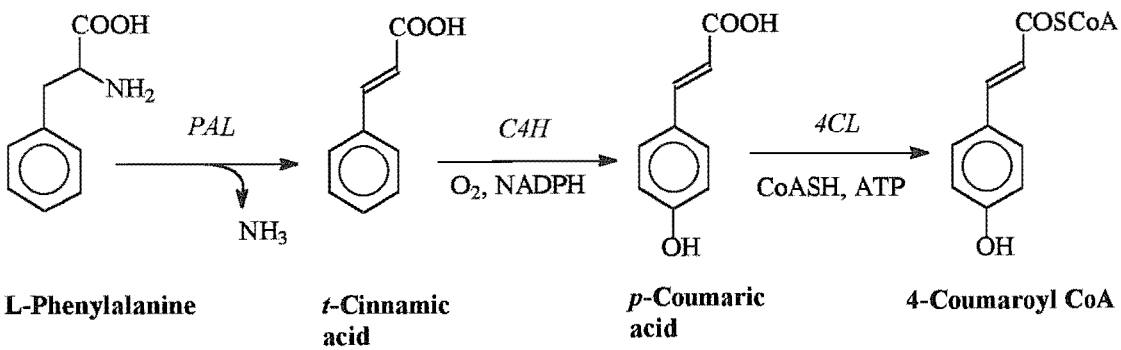


Figure 5.2 Phenylpropanoid pathway (PAL = phenylalanine ammonia-lyase, C4H = cinnamic acid 4-hydroxylase, 4CL = 4-coumarate:CoA ligase).

1) Chalcone synthase (CHS)

The first step which is specific to the flavonoid pathway is the condensation of two molecules of malonyl CoA and one of acetyl CoA with 4-coumaroyl CoA to form the C_{15} intermediate, naringenin chalcone (2',4,4',6'-tetrahydroxychalcone) (Figure 5.3). Formation of this chalcone is catalysed by the enzyme, chalcone synthase (CHS), and is frequently considered to be the rate limiting step of the specific flavonoid pathway (Stafford, 1990). CHS has been purified from a number of plants (see Stafford, 1990 for review). CHS shows end-product inhibition, with CoA, naringenin, eriodictyol, apigenin and luteolin all having been shown to inhibit the activity. The expression of the CHS enzyme and its activity has been found to be light induced (Stafford, 1990).

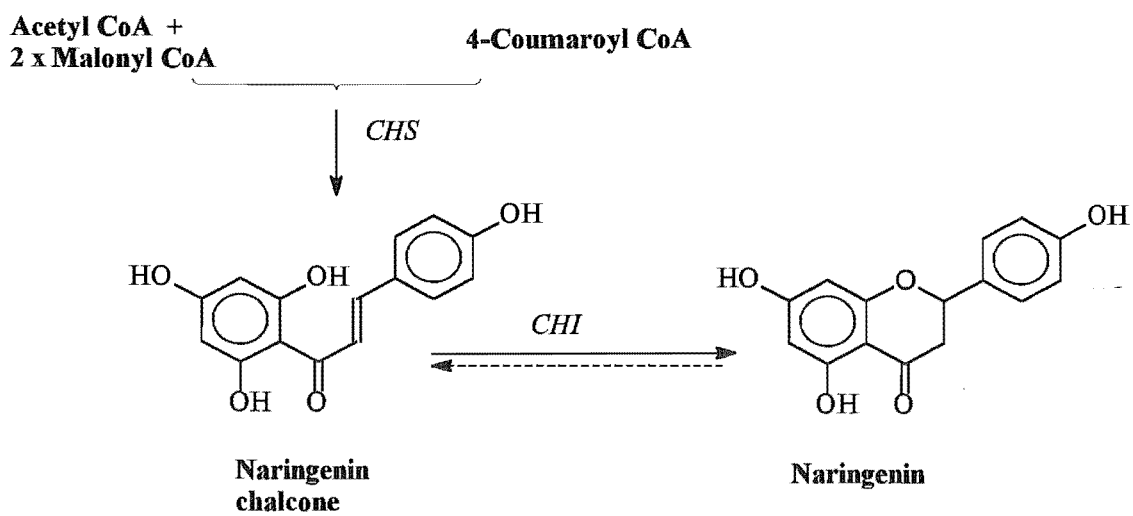


Figure 5.3 Formation of naringenin chalcone and naringenin by chalcone synthase (CHS) and chalcone isomerase (CHI).

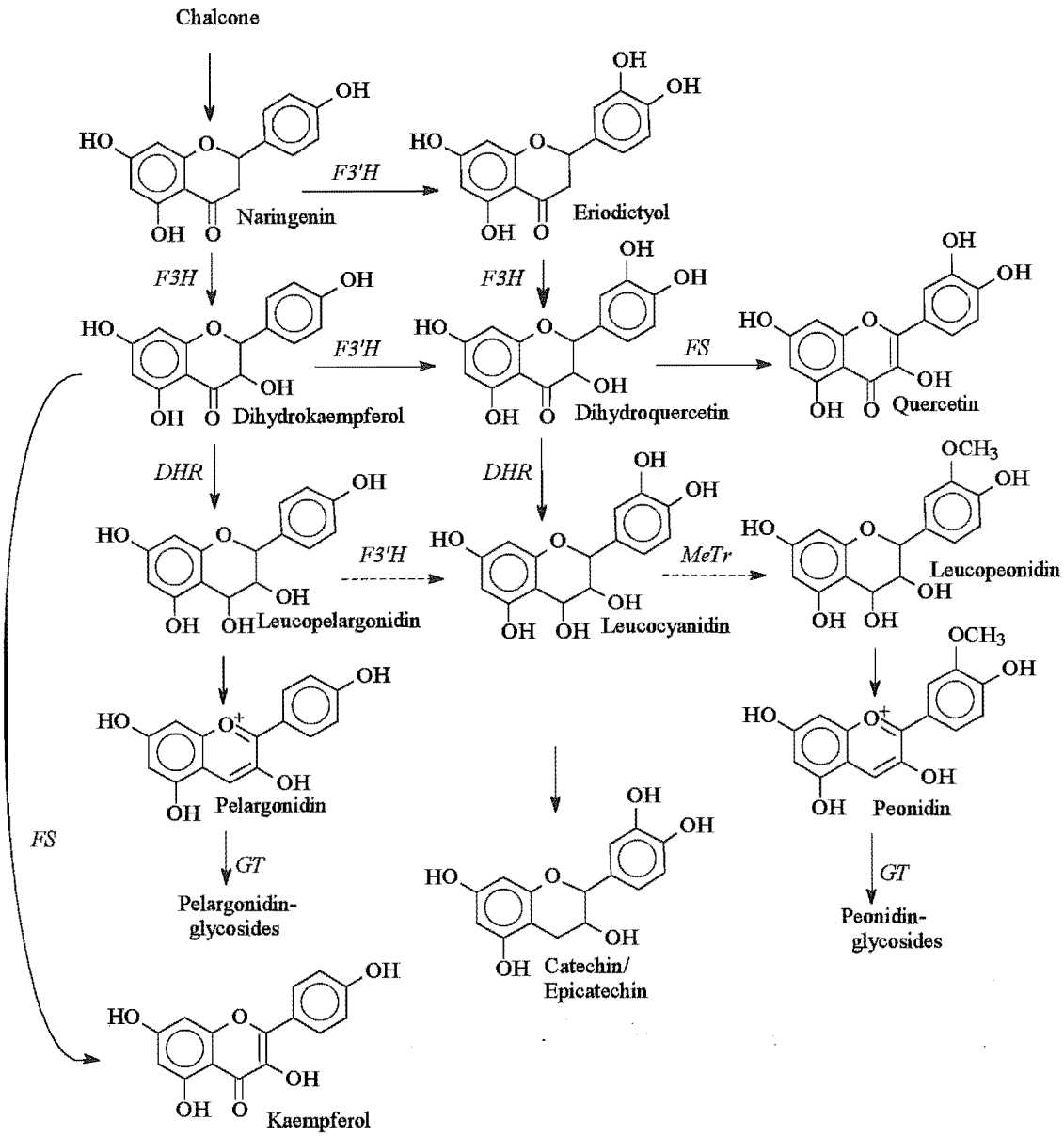


Figure 5.4 Major flavonoids found in the red-skinned tubers of cultivar Desirée, and their inter-relationships. Solid arrows indicate single reactions in the major pathways and broken arrows indicate postulated parallel reactions. (*DHR* = dihydroflavonol reductase, *F3H* = flavanone 3-hydroxylase, *F3'H* = flavonoid 3'-hydroxylase, *FS* = flavonol synthase, *GT* = glycosyltransferase, *MeTr* = methyltransferase).

2) Chalcone isomerase (CHI)

Most classes of flavonoid (e.g. flavonols and anthocyanins) are derived from a flavanone intermediate. The flavanone, naringenin [(-)-(2*S*)-7,4'-dihydroxyflavanone] is formed from naringenin chalcone (Figure 5.3). This reaction, which does occur spontaneously, is catalysed at a faster rate by the enzyme chalcone isomerase (CHI). In contrast to the spontaneous reaction which is not stereospecific, a high degree of stereospecificity has

been found for the enzyme, with 99.99% of the product formed as the 2*S*-flavanone, which is required for further reactions in the flavonoid pathways (Stafford, 1990). Both CHS and CHI activities are induced together under certain conditions (e.g. light), and tend to change in a similar pattern during development (Heller and Hahlbrock, 1980; Stafford, 1990).

Following the formation of naringenin there are a number of different routes towards the formation of different classes of flavonoids, and a general biosynthetic pathway for the flavonoid classes has been shown in Figure 1.3. Because most of these classes have not been found in potatoes, a more detailed insight into the biochemistry of those flavonoids which are produced in potatoes will be discussed. From pathways described by Grisebach (1982), Stafford (1990) and Lister (1994) and in conjunction with the results presented in Chapter 3, a pathway for the biosynthesis of mono- and dihydroxylated flavonoids in red potato tubers (containing only Pg- and Pn-glycosides in the anthocyanin class, e.g. Desirée) is postulated (Figure 5.4). The biosynthesis of flavonoids in purple tubers is similar, but additionally includes the synthesis of trihydroxylated anthocyanins and their methylated derivatives (Dp, Pt and Mv).

The major classes of flavonoids produced in potatoes are flavanones, flavonols, flavan-3-ols and anthocyanins (Chapter 3). Small amounts of flavones have also been reported in some *Solanum* species (Harborne, 1967; Stafford, 1990), but because they were not detected in this study, a detailed discussion of their biosynthesis will not be included.

3) Flavanone 3-hydroxylase (F3H)

Dihydroflavonols are thought to represent a branch point in the biosynthetic pathway of potato flavonoids, with flavonols, flavan-3-ols and anthocyanins all formed from this class of compound. The conversion of flavanones (e.g. naringenin or eriodictyol) to dihydroflavonols (e.g. dihydrokaempferol (dOHKm) or dihydroquercetin (dOHQu)) is catalysed by the enzyme flavanone 3-hydroxylase (F3H) (Figure 5.4). This enzyme has been detected from a variety of plants, and has been characterised as a soluble dioxygenase which requires 2-oxoglutarate, Fe^{++} and ascorbate as co-factors (Britsch and Grisebach, 1986).

4) Flavonol formation

The enzyme flavonol synthase (FS), which converts dihydroflavonols to flavonols, is also a dioxygenase requiring 2-oxoglutarate, Fe^{++} and ascorbate, but has not been studied in as much detail as many of the other enzymes of the flavonoid pathways. FS is able to catalyse the conversion of dOHKm to kaempferol (Km) and dOHQu to quercetin (Qu)

(Figure 5.4), and also dihydromyricetin (dOHMy) to myricetin (My) (Gerats *et al.*, 1982).

5) Flavan-3-ol formation

Flavan-3-ols (catechins) are formed from dihydroflavonols in two steps (Figure 5.4). The first step, catalysed by the NADPH dependent enzyme dihydroflavonol reductase (DHR), is the production of the flavan-3,4-diol (leucoanthocyanidin, e.g. leucocyanidin). The second step is the further reduction of the flavan-3,4-diol (e.g. leucocyanidin) to the flavan-3-ol (e.g. catechin or epicatechin), which is catalysed by 3,4-*cis*-diol 4-reductase (Harborne, 1988). The B-ring of flavan-3-ols generally has a 3',4'-dihydroxyl or 3',4',5'-trihydroxyl pattern; the 4'-hydroxyl pattern which would result from the reduction of dOHKm is not usually found (Stafford, 1990).

6) Anthocyanidin formation

Previously, there has been some controversy over the pathway to anthocyanins however, it has now been demonstrated, by supplementation studies and tracer studies using seedlings and cell suspension cultures, that dihydroflavonols are intermediates in anthocyanin biosynthesis (Stafford, 1990). The participation of flavan-3,4-diols in anthocyanin biosynthesis has also been shown by a series of supplementation experiments in which these precursors were fed to mutant acyanic flowers resulting in anthocyanin production (Heller *et al.*, 1985a and b). Similarly, white *Camellia* flowers which were fed with dOHKm, dOHQu, dOHMy, naringenin and eriodictyol showed a large increase in the production of anthocyanins (Bowen, 1994). Therefore, it is thought that anthocyanin biosynthesis involves the enzyme DHR to convert dihydroflavonols to flavan-3,4-diols (Figure 5.4). However, the enzyme(s) responsible for the conversion of flavan-3,4-diols to anthocyanidins have not yet been discovered, although various possible reaction schemes have been postulated (Heller and Forkman, 1988; Stafford, 1990). Genes (CHS and DHR) involved in anthocyanin biosynthesis have recently been isolated, and these may provide information on the enzyme(s) involved (Gutterson, 1993).

7) B-ring hydroxylation and methylation

The 4'-hydroxyl group of the B-ring is introduced by the incorporation of 4-coumaroyl CoA during the first step of flavonoid biosynthesis. Other B-ring substitution patterns may be determined in two ways: 1) incorporation of already substituted hydroxycinnamic derivatives to the C_{15} skeleton during this first step or, 2) subsequent B-ring substitution of C_{15} intermediates by specific hydroxylases (Heller and Forkman, 1988). 4-Coumaroyl CoA is the usual substrate for CHS but, in some cases caffeoyl CoA and feruloyl CoA can also serve as substrates *in vitro*, but these are typically less

effective (Heller and Forkman, 1988). In contrast, a variety of plant extracts have been shown to catalyse the addition of a hydroxyl group at either the 3' or 5' position of the B-ring at the C_{15} level. These reactions are catalysed by flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3',5'H), which are cytochrome P-450 mono-oxygenases found in microsomal fractions, and require NADPH and O_2. These hydroxylases are non-specific in their recognition of the hydroxylation pattern of the C-ring, and therefore can act on most classes of flavonoid. This results in many species having a similar hydroxylation pattern throughout all classes of flavonoids, and also provides a metabolic "grid" where, for example, dOHQu can be made from two different pathways from naringenin, via either eriodictyol or dOHKm (Figure 5.4) (Stafford, 1990).

Methylation of B-ring hydroxyl groups is also thought to occur at the C_{15} level, and is catalysed by specific *O*-methyltransferases which use *S*-adenosyl-L-methionine (SAM) as the methyl donor.

8) Glycosylation and acylation

Glycosylation and acylation are thought to be late or terminal steps in the biosynthesis of flavonoid glycosides. Two types of glycosides have been found in plants: *O*-glycosides and *C*-glycosides (Heller and Forkmann, 1988). Only *O*-glycosides have been found in potatoes, so glycosylation is catalysed by *O*-glycosyltransferases (GT) which generally transfer a sugar group from its UDP-derivative. Unglycosylated anthocyanidins are rarely, if ever, found in nature because they are usually converted into the more stable 3-glycosides. The glycosylation step converts the flavonoid into a more water soluble constituent which is necessary for the retention of vacuole stored flavonoids. Acylated sugars are common in many flavonoid classes, particularly the anthocyanins, and this reaction is catalysed by acyltransferases. Acylation tends to make the flavonoid more stable because the extent of hydration is reduced (Chapter 2).

5.1.2 Phenolic acid biosynthesis

The phenolic acids are also produced via the shikimic acid and phenylpropanoid pathways. The cinnamic acids are produced from *t*-cinnamic acid via a series of hydroxylation and methylation steps, and the benzoic acids are produced from these cinnamic acids by the alteration of the C_3 side chains to C_1 side chains (Figure 5.5).

5.1.3 Internal regulation of flavonoid biosynthesis

Flavonoid biosynthesis is genetically controlled, and certain plants and tissues are capable of producing only some classes of flavonoids; or can only produce flavonoids with a

certain substitution pattern. However, the types of flavonoid, and especially amounts of these flavonoids, that the plant can synthesise are often regulated by internal and external factors and are produced at different times during plant development (e.g. the "flush" of anthocyanins produced in young seedlings).

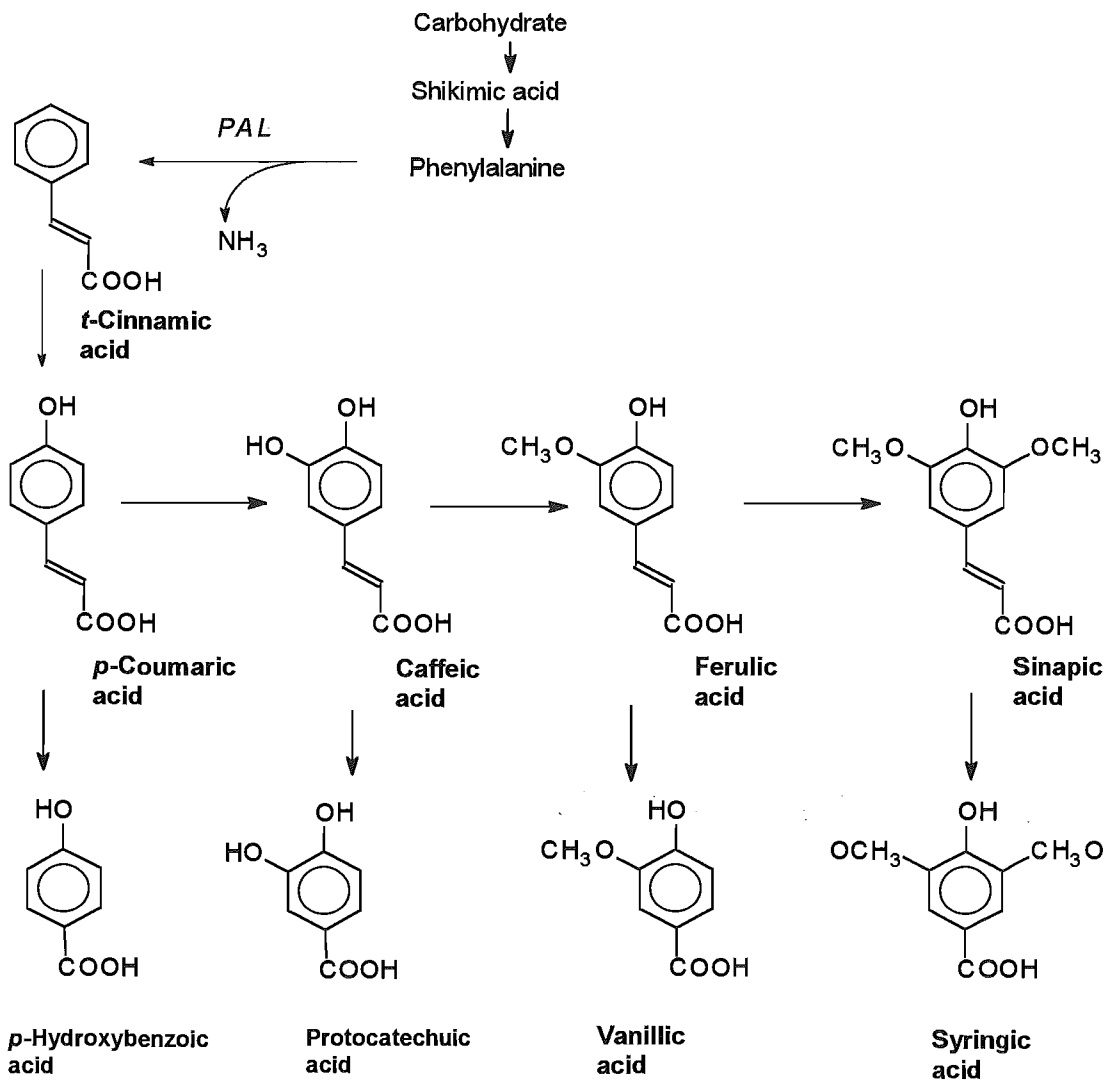


Figure 5.5 Biosynthetic pathway of cinnamic acids and benzoic acids (PAL = phenylalanine ammonia-lyase).

5.1.3.1 Substrate supply

As has been stated previously, PAL is usually considered to be the main limiting factor in the biosynthesis of phenylpropanoids, including anthocyanins, flavonoids and phenolic acids (Section 5.1.1.1). In contrast to this hypothesis is work carried out by Margna

(1977) which suggested that substrate (phenylalanine) supply, rather than enzymatic (PAL) activity, was the most likely limiting factor controlling phenylpropanoid accumulation. This hypothesis is based on their findings which include: 1) the frequent lack of consistent correlation between changes in the levels of PAL and accumulation rate of phenylpropanoids, 2) the high deaminating capacity of PAL markedly exceeds the level required to sustain the formation of polyphenols in most tissues, 3) stimulatory action of phenylalanine on polyphenol accumulation when administered externally, 4) very low and constant levels of free endogenous phenylalanine in plant tissues and, 5) the existence of a balanced relationship between protein metabolism and the formation of flavonoids and cinnamic acid derivatives in plants (Margna, 1977). However, this hypothesis that the limiting factor controlling PAL is substrate supply has not been substantiated by other workers.

5.1.3.2 Sub-cellular location and site of flavonoid biosynthesis

Flavonoid end products are usually accumulated in the large central vacuole, in the cell walls or, in some cases, on the external surface of plant organs such as leaves (Strack *et al.*, 1989). However, although large quantities of flavonoids accumulate in the vacuole, this is not thought to be the site of biosynthesis. It is now thought that flavonoids are synthesised on various parts of the endoplasmic reticulum (ER), in spite of the fact that some of the enzymes are easily solubilized (e.g. PAL, CHS, CHI, F3H, GT), whilst others (e.g. C4H, F3'H, F3',5'H) are not. Although called "soluble" enzymes, these enzymes are only termed "soluble" because most homogenisation and fractionation procedures destroy cellular organisation (Hrazdina and Wagner, 1985a). In fact, these enzymes *in vivo* interact with each other and with structural components in the cell, such as the ER membrane. The "insoluble" C4H, F3'H and F3',5'H enzymes, which are isolated in microsomal fractions, are thought to be located within the ER membrane *in vivo* (Stafford, 1990). Therefore, instead of flavonoid synthesis occurring in the vacuole (where most of the flavonoids are found), it is thought that it occurs in the cytoplasm on the ER membrane. Hrazdina and Wagner (1985a) and Hrazdina (1992) suggested that flavonoid metabolism is facilitated by a multienzyme complex consisting of consecutively assembled, membrane-associated, enzymes (Figure 5.6). Hrazdina and Wagner (1985b) found that PAL and GT, the first and last enzyme of the flavonoid biosynthetic sequence, were located in the lumen of the ER membranes. C4H was found to be membrane embedded, whilst the other enzymes appeared to be weakly associated with the cytoplasmic face of the ER membranes. Hrazdina and Wagner (1985a) suggested that the end products of flavonoid synthesis were accumulated in a membrane enclosed vesicle, and these vesicles could then be transported to the central vacuole for internal sequestration, or to the plasma membrane for secretion of flavonoids (Figure 5.6).

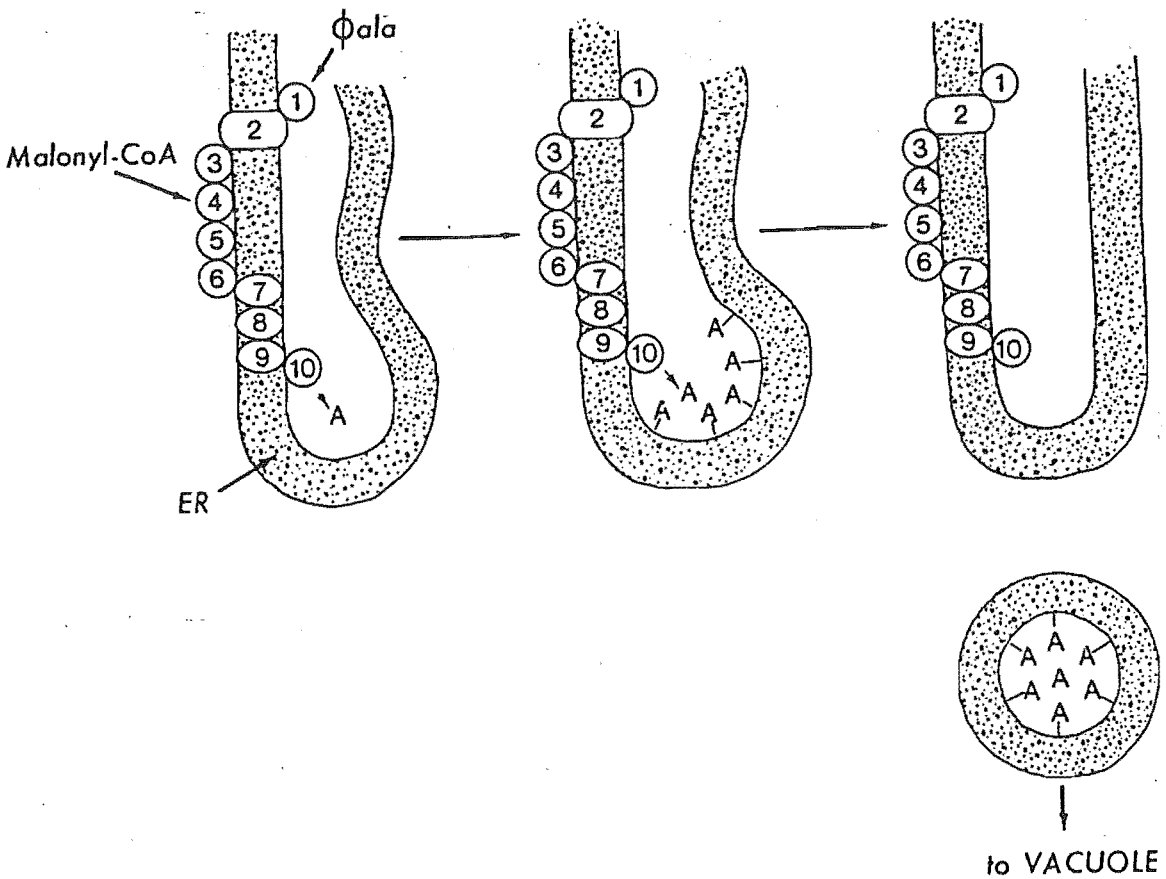


Figure 5.6 Working model of the location of enzymes and biosynthesis of phenylpropanoids, flavonoids and anthocyanins. [Adapted from Hrazdina and Wagner, 1985a] In the synthesis of an anthocyanin - **A**, an endoplasmic reticulum (ER) portion bears an internal phenylalanine ammonia-lyase (PAL) - **1**, that is associated with transmembrane cinnamic acid 4-hydroxylase (C4H) - **2**. C4H channels its product, *p*-coumaric acid, to the cytoplasmic face of the ER where the four consecutive enzymes of the pathway, 4-coumarate:CoA ligase (4CL) - **3**, chalcone synthase (CHS) - **4**, chalcone isomerase (CHI) - **5**, and flavanone 3-hydroxylase (F3H) - **6**, are located. Further transformation of the dihydroflavonol to the anthocyanidin aglycone takes place on the two or three membrane embedded oxido-reductases, dihydroflavonol reductase (DHR) - **7**, and the proposed enzyme(s) to convert the flavan-3,4-diol to the anthocyanidin - **8** and **9**. The anthocyanidin aglycone is glycosylated by a glycosyltransferase (GT) - **10**, that resides on the lumen face of the ER-membrane. In this model, the products are sequestered in specific regions of the ER that are destined to form transport vesicles. Glycosylated flavonoids and anthocyanins are transported to the central vacuole where they accumulate. Phenylpropanoids and unglycosylated flavonoids may be transported by a similar mechanism to the plasma membrane and excreted into cell wall regions, or to the surface of the plant.

5.1.3.3 Plant hormones

The effects of plant hormones on anthocyanin biosynthesis have often been studied using tissue-cultured plantlets. Gibberellic acid (GA_3) has been found to inhibit the synthesis of anthocyanins and other flavonoids in intact plants of *Spirodela* (Furuya and Thimann, 1964), and CHS was inhibited in carrot cell suspension cultures (Hinderer *et al.*, 1984). Kinetin application caused the increase of *de novo* synthesised PAL activity in tobacco callus, accompanied by accumulation of the coumarins, scopoletin and scopolin (Nagai *et al.*, 1994). 2,4-D (an auxin herbicide) inhibited anthocyanin accumulation and mRNA synthesis in carrot cell cultures (Hinderer *et al.*, 1984). The effect of these hormones on the synthesis of anthocyanins may be important because tuberization in potatoes is also at least partially controlled by hormones (Booth, 1963; Cutter, 1978; Moorby, 1978; Krauss, 1985; Stallknecht, 1985).

5.1.4 External regulation of flavonoid biosynthesis

A number of environmental factors influence flavonoid biosynthesis; these include, light, temperature, disease, wounding and nutritional effects (e.g. water and mineral stress). Disease and wounding typically increase the production of flavonoids, and have been discussed in Chapter 3. Anthocyanin accumulation is generally thought to be stimulated by sugars and has been discussed previously in Section 4.1.4. Stress, induced by extremes of temperature, water or minerals, also tend to increase the production of flavonoids, including anthocyanins, but will not be discussed further here (see Dicosmo and Towers (1984) for a review).

5.1.4.1 Effect of light and photo-regulation

Light is probably the most important environmental factor determining anthocyanin biosynthesis because, in most systems, little or no anthocyanin is formed in darkness (Grisebach, 1982; Mancinelli, 1985). In all systems, even in those that can produce some pigment in darkness, exposure to light results in a marked enhancement of the rate of synthesis and total production of anthocyanins and flavonoids (Hahlbrock *et al.*, 1976; Beggs *et al.*, 1986; 1987; McClure, 1986; Brödenfeldt and Mohr, 1988).

There have been numerous reports of photo-control in a variety of different plants (reviewed in McClure, 1975) and, although experimental techniques and light energy levels have been highly variable, most of the responses seem to be explicable on the basis of four classes of photo-reactions: 1) a red/far-red reversible phytochrome system requiring low-energy, 2) a high irradiance response (HIR) which requires $1 Jcm^{-2}$ or

more of either far-red or blue light, 3) photosynthetic involvement for precursor or co-factor production and, 4) complex responses which work indirectly, by such means as enhanced growth hormone synthesis (McClure, 1975).

Photo-receptors

Three photo-receptors are thought to be involved in the regulation of flavonoid metabolism: 1) the phytochrome system, 2) the blue/UV-A light photoreceptor, often called cryptochrome and, 3) an unidentified UV-B receptor (Stafford, 1990). (UV-A is long wavelength UV light between 320nm and 400nm, whereas UV-B is a shorter wavelength UV light between 280nm and 320nm).

1) Phytochrome

Phytochrome is a non-globular protein which can undergo photo-transformation to absorb maximally at either the red (Pr) or far-red (Pfr) regions of the spectrum, with Pfr slowly decaying back to Pr in the dark.

$$P_r \underset{\text{far-red light (730nm)}}{\overset{\text{red light (660nm)}}{\rightleftharpoons}} P_{fr}$$

Although phytochrome is most well known for its red/far-red response, it also absorbs UV-A and blue light (Mancinelli, 1985). Phytochrome is thought to act in some way to affect membrane permeability, which could lead to many metabolic changes, including altered gene expression and enzyme induction (McClure, 1975). Bassim and Pecket (1975) found that promotion of phytochrome controlled anthocyanin biosynthesis in red cabbage seedlings, caused by exposure to red light, was inhibited by the application of membrane stabilisers. It was suggested that light activated phytochrome affects the permeability of the membrane allowing the substrates required for anthocyanin biosynthesis to pass through the membrane.

2) Other receptors

Very little is known about cryptochrome and the UV-B receptor except that cryptochrome is thought to be a flavoprotein (Mancinelli *et al.*, 1991).

Light responses in plants

Much research has been carried out to investigate the effects of varying exposures to different wavelengths on the biosynthesis of anthocyanins (Duell-Pfaff and Wellmann, 1982; Yatsuhashi and Hashimoto, 1985; Arakawa, 1988; Mancinelli *et al.*, 1991).

Two main responses to light have been found:-

1) small amounts of anthocyanin are formed in response to short light exposures (of a few minutes). This type of anthocyanin formation, induced by short light exposures (pulses), is thought to be phytochrome mediated and shows red/far-red light reversibility.

2) the formation of large quantities of anthocyanins in response to prolonged irradiation. This type of anthocyanin formation shows a typical HIR with: a) the full expression of the response requiring prolonged exposure (hours to days) (290nm-750nm), b) the extent of the response being a function of the duration and fluence rate of the irradiations and, c) the response does not show red/far-red light reversibility (Mancinelli, 1985). These HIR's require UV-A, blue, and/or far-red light, and are thought to be mediated by an interaction between phytochrome and cryptochrome. However, the type and degree of interaction between these two photo-receptors varies significantly depending on the species and experimental conditions (Mancinelli *et al.*, 1991).

Mode of action of light induced flavonoid synthesis

How do these photoreceptor responses affect the activities of enzymes involved in flavonoid and anthocyanin biosynthesis? Early experiments (Hahlbrock *et al.*, 1971a; 1976) showed that irradiation of parsley cell suspension cultures with white light resulted in a co-ordinated increase in the activity of the enzymes involved in the flavonoid biosynthetic pathway, from phenylalanine to flavone glycosides. Enzymes of the general phenylpropanoid pathway (PAL, C4H and 4CL) reached maximal activity about 15h after irradiation. The flavonoid biosynthesis enzymes reached their maximum activity several hours later, and it was shown that a number of enzymes not involved in flavonoid metabolism showed no response to the light treatment (Hahlbrock *et al.*, 1971a; 1976). Later experiments showed that, for the enzymes tested, the changes in enzyme activities were caused by changes in activities of the mRNA coding for the enzymes, which was caused by increased mRNA levels (Schröder, 1977; Schröder *et al.*, 1979; Gardiner *et al.*, 1980; Kreuzaler *et al.*, 1983; Kuhn *et al.*, 1984). It was also demonstrated that, for PAL and CHS, these mRNA increases could be attributed to a transient increase in the transcription rates of the relevant genes (Chappell and Hahlbrock, 1984). Similar HIR's and *de novo* synthesis of enzymes were also observed in: 1) potato disks, where illumination triggered accumulation of chlorogenic acid and interrelated increases of PAL and C4H activities (Lamb, 1979), 2) pea shoots, where lignogenesis was induced by illumination and accompanied by increased activities of PAL, C4H and 4CL (Wilkinson

and Butt, 1992), 3) mustard cotyledons, where increases in PAL and CHS activities corresponded with anthocyanin and quercetin accumulation (Brödenfeldt and Mohr, 1988) and, 4) rice seedlings, where anthocyanin content and PAL activities increased dramatically after illumination (Reddy *et al.*, 1994). There are also numerous reports of the simple phytochrome effects of short bursts of light and red/far-red reversible stimulation of PAL activity, and also in some cases CHS and CHI activity (reviewed in Beggs *et al.*, 1986).

Potatoes are one of the few mature plant systems which have been shown to exhibit these responses to light, since most of the other studies have been carried out with tissue-cultured plantlets, cell suspension cultures or seedlings. Studies using potato tubers have focused on the light induced accumulation of chlorogenic acid in tubers (Lamb, 1979; Lamb and Merritt, 1979; Griffiths *et al.*, 1995), or the production of anthocyanin in light grown potato sprouts as a means of identification (Verma *et al.*, 1972; Houwing *et al.*, 1986). The isolation and characterisation of a cDNA clone encoding potato phytochrome has been carried out by Heyer and Gatz (1992).

UV light, especially UV-B irradiation, can cause impairment or inhibition of plant function resulting in the decline of productivity. This is mainly associated with damage to DNA, proteins and plant membranes. It is thought that the photo-regulation of flavonoid biosynthesis by UV light and photoreceptors is to protect the plant's photosynthetic tissues by increasing the production of flavonoids. Flavonoids absorb light around 300nm and are generally localised in the upper epidermal tissues, therefore acting as screening pigments absorbing harmful UV-B radiation (Tevini *et al.*, 1991; Braun and Tevini, 1993; Middleton and Teramura, 1993; Jorgensen, 1994; Strid *et al.*, 1994).

5.1.5. Use of minitubers to study flavonoid biosynthesis

Tissue-cultured minitubers were used in this work to study the effect of light on flavonoid and, in particular, anthocyanin biosynthesis in potato tubers. Potato plantlet tissue-culture and minituber production are well established techniques and have been used in a number of applications:-

1) experimental tools, where plantlets have been used to investigate a number of factors including: a) host plant responses to infection and disease processes (Platt and Bollen, 1993), b) sucrose and starch metabolism (Zrenner *et al.*, 1995), c) disease resistance and generation of genetic variability (Sebastiani *et al.*, 1994), and d) tuberization and effect of hormones (Melis and van Staden, 1984).

2) routine production of seed tubers, which is important in a) the production of disease free seed potatoes for seed certification programmes, b) use as material for international exchange of germplasm and, c) use for medium term conservation of potato germplasm (Wang and Hu, 1982; Lizarraga *et al.*, 1989; Lommen and Struik, 1994; Ranalli *et al.*, 1994). Methods for the mass production of minitubers have been developed (Akita and Takayama, 1994a and b).

General conditions for tissue-culture growth and minituber production have been established, but these may vary slightly between cultivars. Production of potato plants from nodal cuttings is usually carried out in a basic solid agar medium containing sucrose as a carbon source. This procedure of using nodal cuttings is carried out as follows: single stem nodes are excised from the parent plant, surface sterilised if necessary, and the base of the stem placed in a suitable solid media which encourages both root and shoot growth. To initiate tuberization, these plantlets are then transferred to a basic liquid medium with a high sucrose concentration, with the addition of a growth promoting cytokinin, usually benzyladenine (BA), and with a growth retardant/inhibitor of gibberellin biosynthesis (because gibberellin inhibits tuber formation), typically ancymidol, cycocel, paclobutrazol, or tetraclacis (Estrada *et al.*, 1986; Dodds, 1988; Harvey *et al.*, 1991; Alchanatis *et al.*, 1994; Vreugdenhil *et al.*, 1994). Hussey and Stacey (1984) found that ethylene accumulation in growth containers with sealed covers caused stunting of plantlets and inhibition of tuberization; therefore containers with unrestricted gaseous exchange should be used. It has also been found that ethylene prevents the accumulation of both starch and anthocyanins (Mingo-Castel *et al.*, 1976).

5.1.6 Objectives

Experiments in this section were concerned mainly with answering the following related questions:-

1) what effect does light have on the biosynthesis of anthocyanins, flavonoids and phenolic acids in potato tubers?

2) why do potato tubers produce anthocyanins in the dark and how is this biosynthesis controlled?

These questions were answered mostly by using tissue-cultured minitubers, so that light conditions could be altered easily, and the effects on the production of anthocyanins, flavonoids and phenolic acids determined. The change in enzyme activities after light exposure was monitored, and related to the production of the phenolic compounds.

5.2 Materials and methods

5.2.1 Materials

Plant Material

Initial virus-free potato plantlets for tissue-culture (cultivars Amadeus, Bildtstar, Desirée, Red King Edward (RKE) and Urenika) were obtained from the NZ Institute for Crop & Food Research Ltd, Lincoln.

Tissue-culture

Media reagents were obtained from Sigma (USA), except agar which was obtained from Davis (NZ).

Enzyme assays

L-Phenylalanine, cinnamic acid, NADPH, UDP-galactose, UDP-glucose and UDP-xylose were obtained from Sigma (USA). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim (Germany). Flavonoid standards were obtained from sources as in Section 3.2.1.

Synthesis of tetrahydroxychalcone

Naringenin chalcone (2',4,4',6'-tetrahydroxychalcone), the substrate for the CHI assay, was synthesised from naringenin using the method of Moustafa and Wong (1967), by treatment with 50% KOH followed by acidification and recrystallization from aqueous ethanol.

5.2.2 Methods

5.2.2.1 Tissue-culture and analysis of minitubers

Nodal cuttings of existing tissue-cultured potato plantlets of the cultivars Amadeus, Bildtstar, Desirée, RKE and Urenika were multiplied *in vitro* on Murashige and Skoog salts and vitamins (Murashige and Skoog, 1962), plus 30g/l sucrose, 40mg/l ascorbic acid, 500mg/l casein hydrolysate and 6g/l agar (potato multiplication medium - PMM). Tubers were initiated by transferring the plantlets to liquid media containing the same constituents, except with the addition of 80g/l sucrose (instead of 30g/l), 5mg/l benzyladenine (BA) and 2.5mg/l ancymidol, and the omission of agar (tuber initiation medium - TIM), following the method of Conner *et al.* (1991). All media were sterilised by autoclaving at 103kPa for 20min. Cultures were incubated in plastic pottles (90mm diameter x 60mm high), 50ml medium and ten plants per pottle, at 26°C under cool

white fluorescent lights (150Watts/m^2 (~80μmol/m^2/sec)) with a 16h photoperiod, unless noted otherwise. Cultures were subjected to various light treatments. All plants were grown for two weeks in PMM before nine weeks incubation in the TIM.

Minitubers were too small to be peeled for the measurement of anthocyanins in the skin, so the surface area was approximated by an ellipsoid as described in Chapter 4. This was appropriate for the measurement of anthocyanin concentrations because no anthocyanins were found in the flesh of minitubers, even in Urenika minitubers. However, because the flesh also contained flavonoids and phenolic acids (albeit very small concentrations), and presumably low activities of enzymes, there was the potential for error to occur in the measurement of flavonoid and phenolic acid concentrations and enzyme activities. For these reasons, it was not feasible to measure the enzyme activities of minitubers while they were developing (and a wide range of sizes were found), nor grown in different light treatments as this markedly affected the size of the minituber and it was considered that measurement would reflect the changes in the size of tubers rather than actual changes in enzyme activities. Accordingly, to overcome these problems, minitubers of a similar size were used for all samples for measurement of enzyme activities, with numerous minitubers in each sample (20-30).

Most of the experiments with minitubers were carried out using Desirée plantlets, but cultivars Amadeus, Bildtstar, Red King Edward and Urenika were also used for comparison. Plantlets were grown either in the light (LG) or dark (DG) for two weeks, and then transferred to TIM and kept in either the light (LT) or dark (DT) for nine weeks. Other experiments involved the use of light boxes with coloured filters or shade cloth to decrease the light intensity. For all experiments, except those using full light, the transfer of plantlets to TIM was carried out under green light, using a glass bead steriliser instead of a flame for instrument sterilisation (so that there were no other sources of light). Minitubers were classified as a "minituber" when they were greater than 2mm in diameter.

5.2.2.2 Extraction and analysis of phenolics

Phenolics were extracted in 15% acetic acid in methanol (Section 3.3.2), and analysed by analytical HPLC (Section 3.3.3.2), or by spectrophotometry (Section 4.2.2).

5.2.2.3 Extraction of enzymes

Whole minitubers (a total of about 10g) were ground to a course powder in liquid N_2 with a mortar and pestle. The powder was extracted with ~50ml 50mM Tris-HCl buffer

pH 8, containing 50% (w/w of tuber) washed polyvinyl-polypyrrolidine (PVPP, PolyClar AT), 1mM phenylmethylsulfonylfluoride (PMSF) and 10mM 2-mercaptoethanol, ground further in an Ultra Turrax homogeniser, then the homogenate filtered and centrifuged at 12 000g for 10min at 4°C. The supernatant was used as a source of crude enzyme.

Further purification of enzyme extracts

Acetone precipitation. To half of the crude enzyme extract, three volumes of cold acetone were added sequentially and stirred for 15-20 minutes. The precipitated protein was centrifuged at 12 000g for 10min and redissolved in a small volume of extraction buffer (5-10ml). All procedures were carried out at 4°C.

Microsomal preparation. The other half of the crude enzyme extract was used for microsome preparation, where 1M $MgCl_2$ was added to give a final concentration of 30mM $MgCl_2$ (Diesperger *et al.*, 1974). After thorough mixing, the precipitated microsomes were centrifuged at 12 000g for 10min and redissolved in a small volume of extraction buffer (2-3ml). All these procedures were carried out at 4°C.

5.2.2.4 Estimation of protein concentration

Protein was estimated by the dye-binding method of Bradford (1976), using bovine serum albumin (BSA) as a standard. Samples were usually diluted five-fold with 50mM Tris-HCl buffer pH 8, to give readings in the linear range of the standard curve, which was measured each time.

5.2.2.5 Assay of PAL activity

PAL activity in the acetone precipitate was assayed by a continuous spectrophotometric method. The assay mixture (500µl) contained 100mM Tris-HCl buffer pH 8 (250-345µl) and acetone precipitated enzyme extract (5-100µl), equilibrated in a cuvette at 30°C. The reaction was initiated by the addition of 20mg/ml L-phenylalanine (150µl to give a final concentration of 6mg/ml), and the rate of cinnamic acid production measured by the increase in absorbance at 290nm over a 1-2h period, depending on the activity. Duplicate assays were performed for each extract, both with and without substrate, at three to five concentrations of enzyme extract, to ensure that the assay was measured within the linear range of enzyme activity and cinnamic acid absorbance.

5.2.2.6 Assay of C4H activity

Assay of C4H activity was carried out on the microsomal enzyme precipitate. The 500µl assay mixture contained 100mM phosphate buffer (Na_2HPO_4/KH_2PO_4) pH 7.5, 1mM dithiothreitol (DTT), 1mM NADPH and up to 250µl enzyme extract. The reaction was initiated with the addition of 10mM *t*-cinnamic acid (final concentration 1mM) and the mixture, in open Eppendorf tubes, was incubated at 30°C for 60min with frequent shaking for aeration. The reaction was stopped by the addition of 50µl acetic acid, and tubes were centrifuged at 20 000g for 20min to pellet the denatured protein. The production of *p*-coumaric acid was measured by analytical HPLC, for two concentrations of the microsomal enzyme extract.

5.2.2.7 Assay of CHI activity

CHI activity was measured in a continuous spectrophotometric assay. The assay mixture consisted of 200mM Tris-HCl buffer pH 7.4 containing 50mM KCN and 10mg/ml BSA (total 422.5µl) and 75µl acetone precipitated enzyme extract (diluted from 0 to 40 fold, depending on enzyme activity). The reaction was initiated with the addition of 2.5µl 10mg/ml naringenin chalcone in 2-ethoxyethanol (Section 2.5.l). Absorbance was measured at 380nm for 10min with the cuvette maintained at 30°C. Control reactions contained the assay mixture without enzyme, to account for the spontaneous isomerization of the chalcone. The initial rate of disappearance of the chalcone in the presence of enzyme was used to estimate CHI activity. All samples were assayed in duplicate (including control) at three to four concentrations of enzyme, to ensure that assays were measured in the linear range of enzyme concentration. Controls without the addition of substrate were also carried out.

5.2.2.8 Assay of reductase (DHR) activity

This assay measured a double reaction; 1) the activity of DHR and 2) the activity of the diol-reductase involved in the synthesis of anthocyanins and flavan-3-ols, by adaptation of the methods of Stafford and Lester (1982; 1984; 1985). The reaction mixture (500µl) contained 50mM Tris-HCl buffer pH 7.5, 1mM NADPH, 6mM glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 1mM substrate (usually dOHQu) and acetone precipitated enzyme extract (usually 100µl), and was incubated at 30°C for 90min. The reaction was stopped with the addition of 50µl acetic acid and centrifuged at 20 000g for 20min before analysis by analytical HPLC.

5.2.2.9 Assay of F3H activity

The reaction mixture (500µl) contained 100mM Tris-HCl buffer pH 7.5, 0.5mM DTT, 0.25mM 2-oxoglutarate, 0.05mM ferrous sulphate, 5mM sodium ascorbate, 0.5mM substrate (eriodictyol or naringenin) and 50-75µl acetone precipitated enzyme extract. The reaction was initiated by the addition of substrate and incubated at 30°C for 60min. The reaction was stopped with 50µl acetic acid, centrifuged at 20 000g for 20min, and the analysis of products was carried out by analytical HPLC.

5.2.2.10 Assay of F3'H and F3',5'H activity

F3'H and F3',5'H are microsomal enzymes, so assays were carried out using the microsomal preparations. The 500µl assay mixture, in open Eppendorf tubes, contained 200mM phosphate buffer (Na_2HPO_4/KH_2PO_4) pH 7.5, 0.5mM DTT, 0.4mM NADPH, 0.2mM substrate (*p*-coumaric acid, naringenin, Km or apigenin) and 50-75µl enzyme extract. The reaction was initiated with the addition of substrate and incubated at 30°C for 60min with frequent shaking for aeration. The reaction was stopped with the addition of 50µl acetic acid, centrifuged at 20 000g for 20min, and the products analysed by analytical HPLC.

5.2.2.11 Assay of GT activity

The 200µl reaction mixture contained 50mM Tris-HCl buffer pH 8, 0.25mM substrate (Km, Qu or luteolin), 1mM UDP-sugar (glucose, galactose or xylose) and 50-75µl acetone precipitated enzyme extract, and was incubated for 60min at 30°C. The reaction was stopped with the addition of 20µl acetic acid, centrifuged at 20 000g for 20min, and the products analysed by analytical HPLC.

5.2.2.12 HPLC analysis of enzyme reactions

Analysis of enzyme assay products by analytical HPLC was carried out by the method described in Section 3.3.3.2.

5.2.2.13 Presentation of results and statistical analysis

Results for enzyme analyses were presented as a percentage of the highest activity (mmol product produced/min/mg protein) for that particular enzyme. Activities were presented in this manner rather than actual units, because it enabled the changes of activity of different enzymes to be directly compared. Additionally, it was found that enzyme

activities were generally a number of times higher in the skin than in the flesh, but whole minitubers (of similar sizes) were used for analysis (with no separation of skin and flesh tissues), and it was felt that to report units would be misleading.

Regression analyses comparing enzyme activities and anthocyanin, flavonoid or phenolic acid concentrations were carried out using the computer program CoStat (Berkeley, USA). Curves were fitted to the graphs using the polynomial regression analysis in CoStat, except for Figures 5.14, 5.16, 5.18-5.23 where curves were fitted by eye.

5.3 Preliminary development and evaluation of enzyme assays

This section is presented to explain the development of the methods used for the enzyme assays carried out in this chapter, and also the optimisation and evaluation of these methods. A number of chromatographic and spectrophotometric assays were investigated for the measurement of enzyme activities because these were quicker and cheaper than the HPLC methods. However, in most cases the activity in potato tubers was not high enough to use these chromatographic or spectrophotometric methods (except PAL and CHI), and the more sensitive HPLC methods were used for all further analyses. These other methods are still presented here, even when they were not used later, because they may be of use for future research in the assay of plants with higher activities of these enzymes.

5.3.1 Enzyme extraction

Enzymes were originally extracted in 100mM sodium borate buffer pH 8.8, containing 2mM DTT and 10mM PMSF. DTT is a stereoselective reducing agent for the disulphide bridges in complex molecules and was not sufficient to prevent oxidation and browning of the extract. Therefore, PVPP was added to remove the phenolics which act as substrates for the enzymatic browning reaction and may also interfere with later enzyme assays, and 2-mercaptoethanol (10mM) was added to replace the DTT to prevent oxidation and browning of the extract. These additions prevented the browning reactions and markedly increased the enzyme activities. The sodium borate buffer was replaced with 100mM Tris-HCl buffer pH 8.0, so that one extraction buffer could be used for all enzymes. PMSF was retained to inhibit serine proteases (e.g. trypsin and chymotrypsin). The combination of PVPP in the extraction buffer, and acetone or $MgCl_2$ precipitation produced an enzyme extract which, when checked by HPLC, did not contain any phenolics, and contained only a few very early eluting compounds (2-4min) which were probably low molecular weight peptides (K.Sutton, pers. comm.). Although

a lipid solubilising agent (such as Triton X-100) is often included in the extraction buffer for the extraction of GT (Heinsbroek *et al.*, 1979), it was not included in this case because there was no significant increase in GT activity upon its addition, and it decreased the activity of some of the other enzymes. Ammonium sulphate precipitation was also considered as an alternative to acetone precipitation, but activity for some enzymes (particularly CHI) was reduced by this method, probably because of the overnight dialysis step required for ammonium sulphate removal, because CHI activity had a short half-life.

5.3.2 Purification of microsomes

C4H and F3'H activities were carried out using microsomal extracts, and two methods were investigated for the purification of these microsomes.

1) The ultra centrifugation method, based on the methods of Russell (1971) and Büche and Sandermann (1973) where the microsomes were precipitated from the crude extract (not the acetone extract) by ultra centrifugation at 100 000g for 60min, at 4°C, and redissolved in a small volume of extraction buffer.

2) The $MgCl_2$ precipitation method of Diesperger *et al.* (1974), which involved the precipitation of microsomes from the crude extract by the addition $MgCl_2$ as in Section 5.2.2.3.

Both these methods were used to precipitate microsomes from a number of samples, and the C4H and F3'H activities of the resulting microsomes (redissolved in a small volume of extraction buffer) were assayed as in Section 5.2.2. For both these enzymes the highest activity (per mg protein) was shown by microsomes precipitated by the $MgCl_2$ method, so this method was used for the preparation of microsomes in all further experiments.

5.3.3 Phenylalanine ammonia lyase assay

PAL activity was measured by a continuous spectrophotometric assay in which the production of cinnamic acid was measured by the increased absorbance at 290nm. The calibration curve of cinnamic acid absorbance was linear up to 20μg/ml, with a R^2 value of 99.7%. PAL activity was originally measured in 100mM sodium borate buffer pH 8.8, but activity was increased and more stable in 100mM Tris-HCl buffer pH 8.8. To check the effect of increasing enzyme concentration on the measured enzyme activity, the amount of added enzyme was increased from 25 to 250μl (Figure 5.7). This showed a linear increase in activity up to 200μl, after which the activity decreased.

It was thought initially that this decrease may have been caused by the inhibitor, PAL inactivating system (PAL-IS) which has been found in some plant extracts, including potato tubers (Zucker, 1968), sunflower leaves (Creasy, 1976), sweet potato tubers (Tanaka *et al.*, 1977) and apple skin and leaves (Tan, 1979), and is thought to be a proteolytic enzyme (Hanson and Havir, 1981). To determine if this decrease was caused by PAL-IS, PAL-IS was extracted and assayed by the method of Tan (1979). Potato samples were ground in liquid N_2, and 50% PVPP and 100mM MES buffer (pH 5.5) were added. The homogenate was centrifuged at 20 000g for 15min. Sodium borate was added to the supernatant until the pH reached 8.8, and then centrifuged at 5000g for 10min to remove any excess sodium borate which may have remained undissolved. This final supernatant, which did not contain PAL activity, was used immediately for assay of PAL-IS. This was carried out by the addition of the PAL-IS extract (from potatoes) to an enzyme extract from both *Camellia* flowers (high PAL activity) and potato tubers, which was incubated for 15min at 30°C before being assayed for PAL activity. The control consisted of only the PAL extract (from *Camellia* flowers or potato tubers) and 100mM sodium borate buffer, pH 8.8.

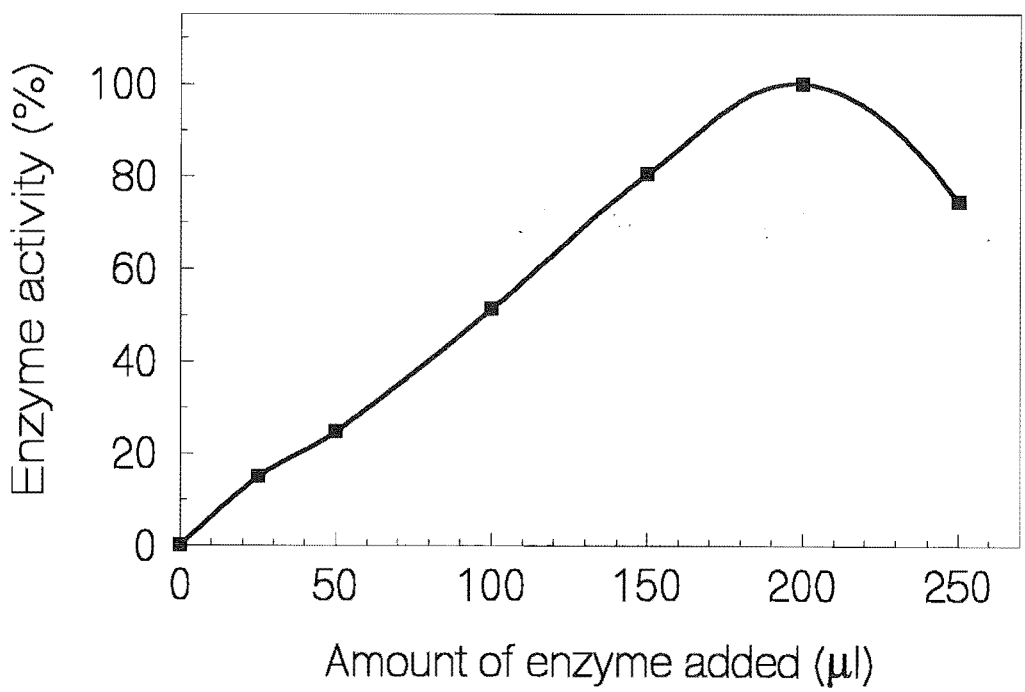


Figure 5.7 Effect of enzyme concentration on PAL activity.

There was a small, but non-significant, decrease in PAL activity with the addition of PAL-IS, and therefore it was decided that, although PAL-IS may be present in small quantities in potato tubers, and may have caused the comparative decrease when 250µl was used (Figure 5.7), it was not present in sufficient quantities to warrant measuring throughout the following experiments. Also, since only 5 to 100µl enzyme extract was used for PAL assays, the effect of PAL-IS would be further minimised.

The effect of altering the phenylalanine concentration was also investigated and it was found that increasing phenylalanine concentration resulted in a linear increase in PAL activity. The substrate concentration could not be optimised because of the low solubility of phenylalanine, so the highest concentration at which phenylalanine remained soluble in the stock solution was used (6mg/ml final concentration).

A calibration curve for cinnamic acid absorbance (not shown) gave a linear relationship between cinnamic acid concentration and absorbance at 290nm up to 25µg/ml, after which the curve tailed off.

During assays, PAL activity tended to show a slight lag for the first 15min, followed by a linear period with a subsequent decrease after 2-3h, therefore rates were usually determined from the linear 30-60min period.

5.3.4 Chalcone isomerase assay

CHI activity was measured by a continuous spectrophotometric assay in which the decrease in absorbance of naringenin chalcone at 380nm was measured. The substrate, naringenin chalcone, underwent a bathochromic spectral shift when alkali was added. Because the optimal pH for CHI (pH 7.4) is in the middle of this shift, the pH of this assay must be tightly controlled to avoid absorbance changes caused by changes in pH rather than enzyme activity. Additionally, naringenin chalcone was stable in 2-ethoxyethanol, and in very acidic or basic solutions, but in aqueous solutions at other intermediate pH's naringenin chalcone showed a spontaneous decrease in absorbance at 380nm. This is caused by the non-enzymatic cyclisation of the chalcone and occurs maximally between pH 7 and 8 (Figure 5.8). The addition of BSA at a final concentration of 10mg/ml markedly reduced the change in absorbance caused by the spontaneous decrease of the substrate at pH 7.4 (not shown). This depression of the self-cyclisation of the chalcone by the addition of BSA at pH values between 5.5 and 9.0 was also shown by Mol *et al.* (1985). In this CHI assay the addition of KCN was necessary to inhibit the enzyme peroxidase, which can catalyse extremely rapid oxidation

of chalcones, and which would otherwise interfere with measurement of CHI activity (Boland and Wong, 1976; Wilson and Wong, 1976; Dixon *et al.*, 1982).

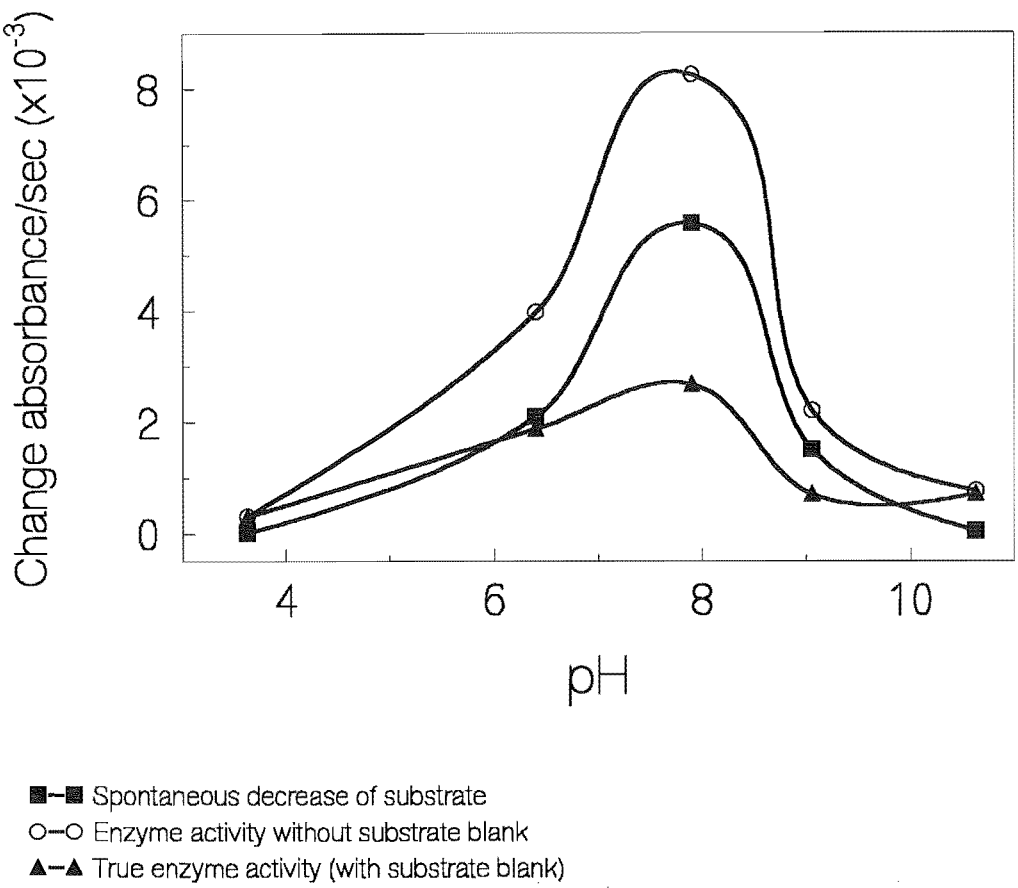


Figure 5.8 Effect of pH on tetrahydroxychalcone and CHI activity, as measured by absorbance at 380nm.

For the assay of CHI activity in the following experiments, both BSA and KCN were included the assay mixtures to minimise self-cyclisation of the chalcone and to ensure the change in absorbance was due solely to CHI activity. Increased enzyme concentrations showed a linear relationship with CHI activity (R^2=99.5%). CHI was found to be moderately unstable, with a half-life of 4-5h, so activity was always measured as soon as practicable after it had been extracted, and all procedures were carried out at 4°C, to minimise loss of activity.

5.3.5 Dihydroflavonol reductase assay

DHR activity was measured in an assay system containing NADPH and the NADPH-regenerating system, glucose-6-phosphate and glucose-6-phosphate dehydrogenase. Initially, tubers from Red Flesh and Urenika cultivars were used as sources of enzyme, with dOHQu as the substrate. Leucoanthocyanidin and catechin products were extracted from the reaction mixture into ethyl acetate and then assayed by either 1) addition of 1ml 4% (w/v) vanillin in methanol plus 0.5ml conc. HCl, incubation in the dark for 15min and the absorbance recorded at 500nm or, 2) the ethyl acetate extract was spotted onto a TLC plate, developed in BAW, and compounds detected with 5% vanillin-HCl in ethanol, after heating at 90°C for 5-10min. However, no reaction was detected using either method. It was thought that this may have been because dOHQu was used as the substrate. The expected products of DHR activity using dOHQu were Cy and/or catechin, however, Cy was not found in tubers (Chapter 3) (although catechin was found), so it was proposed that the tuber DHR could not utilise dOHQu as a substrate. To test this hypothesis dOHKm was used as a substrate for Red Flesh extracts (because they contained Pg) and dOHMy was used as a substrate for the Urenika extracts (because they contained Dp, Pt and Mv). However, these assays also showed no activity using the above detection systems, so the reaction was subsequently analysed by analytical HPLC, which was more sensitive, and activity was then detected in most extracts, using all three substrates. With dOHQu as the substrate, catechin, epicatechin and two unknown products were formed, possibly the flavan-3,4-diol (leucocyanidin) and a proanthocyanidin, but no anthocyanins were detected. This may have been because there were none formed, or because they polymerised immediately to form a proanthocyanidin, or because they were unstable at the neutral pH of the reaction buffer and degraded before the mixture was analysed. This meant that it was not possible to distinguish between the DHR and diol reductase activities in this assay. This would have been possible by supplying the flavan-3,4-diol (leucoanthocyanidin) instead of the dihydroflavonol to measure only the diol reductase, but the lack of commercial availability meant that this had to be synthesised from the dihydroflavonol (see Stafford and Lester (1982) for method), but this was not carried out. Therefore, this assay measured total flavonoid reductase activity, that is dihydroflavonol and diol reductases.

5.3.6 Flavanone and flavonoid hydroxylase assays

Two F3H reactions were assayed:-

naringenin	→	dihydrokaempferol
eriodictyol	→	dihydroquercetin.

Four F3'H reactions were assayed:-

p-coumaric acid	→	caffeic acid
naringenin	→	eriodictyol
kaempferol	→	quercetin
apigenin	→	luteolin.

Initially, it was necessary to determine suitable detection systems, and the following experiments were designed to investigate this. Various TLC methods were tested with the substrates and the products run in different solvents (BAW, Forestal, and 15% acetic acid), and sprayed with different spray reagents to ascertain if products and substrates could be easily distinguished (Table 5.1).

Table 5.1 Chromatographic data for flavanone and flavonoid hydroxylase assays. (* the most effective solvent(s) for distinguishing substrate^S and product^P, ND = not determined, - = no colour).

Substrate^S/ product^P	Rf Forestal	Rf BAW	Rf 15% HOAc	Colour UV	Colour NA-reagent	Colour $AlCl_3$
F3H						
naringenin^S	90.4	95.5	38.8	purple	orange	green
dOHKm^P	ND	ND	ND	ND	ND	ND
eriodictyol^S	80.1	90.1*	34.1*	purple	pink	yellow
dOHQu^P	75.7	70.7*	1.8*	purple	orange	ND
F3'H						
p-coumaric acid ^{S}	93.4*	95.5	32.2	blue	blue	-
caffeic acid^P	72.8*	82.6	30.0	blue	blue	-
naringenin^S	90.4*	95.5	38.8	purple	orange	green
eriodictyol^P	80.1*	90.1	34.1	purple	pink	yellow
kaempferol^S	53.7*	89.4*	2.9	purple	orange	yellow
quercetin^P	39.0*	70.1*	2.4	purple	orange	yellow
apigenin^S	75.7*	93.9*	3.5	purple	orange	yellow
luteolin^P	54.4*	83.6*	3.5	yellow	orange	yellow

Most of the substrates and products could be separated easily by one or two of the solvent systems. NA-reagent proved to be the best spray reagent for the detection of compounds, and distinguishing them by their colour reactions. Although these TLC methods would, in theory, be able to determine if a product was formed, the results

would not be quantitative. Therefore quantitative methods using the diode-array spectrophotometer were investigated. The spectra of the substrates and products (from 250 to 500nm) were recorded in methanol only and after the addition of NA-reagent or $AlCl_3$, and the results are shown in Table 5.2.

Table 5.2 Spectral data (λ_{max} values (nm)) for flavonoid hydroxylase assays. (sh = shoulder, ND = not determined, n.p. = no peak detected between 250-500nm).

Substrate^S/ product^P	λ_{max} values (nm)									
	Methanol			NA-reagent			5% $AlCl_3$			
F3H										
naringenin^S	290	324sh		334			310	376sh		
dOHKm^P	290	330sh		ND			ND			
eriodictyol^S	290	324sh		324			316	376		
dOHQu^P	288	324sh		324sh			314	380		
F3'H										
p-coumaric acid^S	302sh	310		n.p.			318			
caffeic acid^P	302sh	322		356			266	322sh	368	
naringenin^S	290	324sh		334			310	376sh		
eriodictyol^P	290	330sh		330			316	376		
kaempferol^S	264	324sh	366	318sh	366	426	270	300sh	350	424
quercetin^P	256	306sh	366		420	470	272			454
apigenin^S	266		338	312	352	396sh	278	346	382	
luteolin^P	254	294sh	348		334sh	446	274	300sh	332sh	422

For a method to be able to be used for the measurement of activity, the spectra of the two compounds have to be different enough to enable a reading at a particular wavelength to have no absorbance for one compound but a significant absorbance for the other (ideally at or close to the λ_{max}). That is, for the peaks of the two compounds not to significantly overlap. Therefore, the spectra of substrates and products in methanol, with and without colour reagents, were observed to determine if any useful differences between substrate and product spectra existed. The values presented below (in text) indicate the wavelength at which there is minimal overlap of spectra, not necessarily the λ_{max} values for the compound. From the observation of these spectra it was found that *p*-coumaric acid and caffeic acid could be distinguished in NA-reagent at 356nm and in $AlCl_3$ at 370nm, Km and Qu in NA-reagent at 480nm and $AlCl_3$ at 470nm, and apigenin

and luteolin in NA-reagent and $AlCl_3$ both at 450nm, whilst substrates and products from the other reactions could not be distinguished by these methods.

Using these spectrophotometric methods the F3H and F3'H assays were tested using Urenika enzyme extracts, and the products extracted in ethyl acetate. From this ethyl acetate extract, 50µl was added to methanol and the spectrum recorded with and without NA-reagent and $AlCl_3$; and also part of this extract was analysed by TLC. By the use of these spectrophotometric and TLC methods, activity was found to occur only for the following two F3'H assays; *p*-coumaric acid to caffeic acid, and Km to Qu. Analysis by HPLC was more sensitive and activity was found for both these reactions, and all the other F3'H and F3H reactions tested. Therefore, the determination of F3H and F3'H activities was carried out by HPLC in all further experiments. During the course of measurement of F3'H activity using Km as a substrate, both Qu and My were found as products. This suggested that there was some F3',5'H activity present as well as F3'H activity, and that Qu was hydroxylated at the 5'-position to form My. Therefore, in the calculation of F3'H activity, the concentrations of Qu and My were summed to give the total product, as this represented the total F3'H activity. F3',5'H activity was not presented separately.

5.3.7 C4H assay

Initial investigation of C4H activity was based on the spectrophotometric method of Lamb and Rubery (1975), and the reaction mixture was as described in Chapter 5.2.2. For initial experiments, 33µl 6M-HCl was added to the reaction mixture after incubation, and this acidified mixture left to stand for at least 1min to stop the reaction and decompose the NADPH. Phenolics were extracted twice with 500µl ethyl acetate and then the combined extracts were extracted with 500µl 1M-NaOH. The amount of *p*-coumaric acid produced was determined from the absorbance at 330nm. This assay was based on the fact that in the presence of alkali, *p*-coumaric acid has a peak at 330nm, not shown by cinnamic acid, so this provides a useful spectrophotometric system to measure C4H activity. This peak at 330nm was seen only when *p*-coumaric acid was under alkaline conditions.

The efficiency of extraction of *p*-coumaric acid with ethyl acetate and diethyl ether were compared. When the absorbance of *p*-coumaric acid was measured there was a greater than 100% recovery when ethyl acetate was used, whereas when using diethyl ether only 85% of the *p*-coumaric acid was recovered. The greater than 100% recovery using ethyl acetate was likely to be because of the slightly different absorbance properties of *p*-coumaric acid in ethyl acetate, and this was corrected for in the calibration curve. The

decomposition of NADPH in acid was confirmed by the complete disappearance of the NADPH peak at 330nm after 1min.

5.3.7.1 Effect of pH on p-coumaric acid spectra

pH had a marked effect on the spectrum of *p*-coumaric acid, especially on the peak at 330nm (Figure 5.9). Absorbance (at 330nm) increased sharply from pH 8 to 11.5 and then decreased again at higher pH's (Figure 5.10). Therefore, because the absorbance of *p*-coumaric acid was so pH sensitive, a 0.5M phosphate/NaOH buffer at pH 11.5 was used instead of 1M-NaOH which was suggested by Lamb and Rubery (1975).

A calibration curve for *p*-coumaric acid absorbance was constructed, where solutions of each concentration of *p*-coumaric acid were acidified, extracted with ethyl acetate and then extracted with the pH 11.5 buffer, because the ethyl acetate extraction caused the over-estimation of *p*-coumaric acid. This gave a linear calibration curve with the equation of $y = 0.013x$, and a R^2 value of 99.5% (not shown).

When potato tubers were assayed no activity was detected by this spectrophotometric method, even when a number of the reagents and conditions were altered. Therefore, it was decided to analyse the reaction by HPLC which was more sensitive, and activity was detected in most samples.

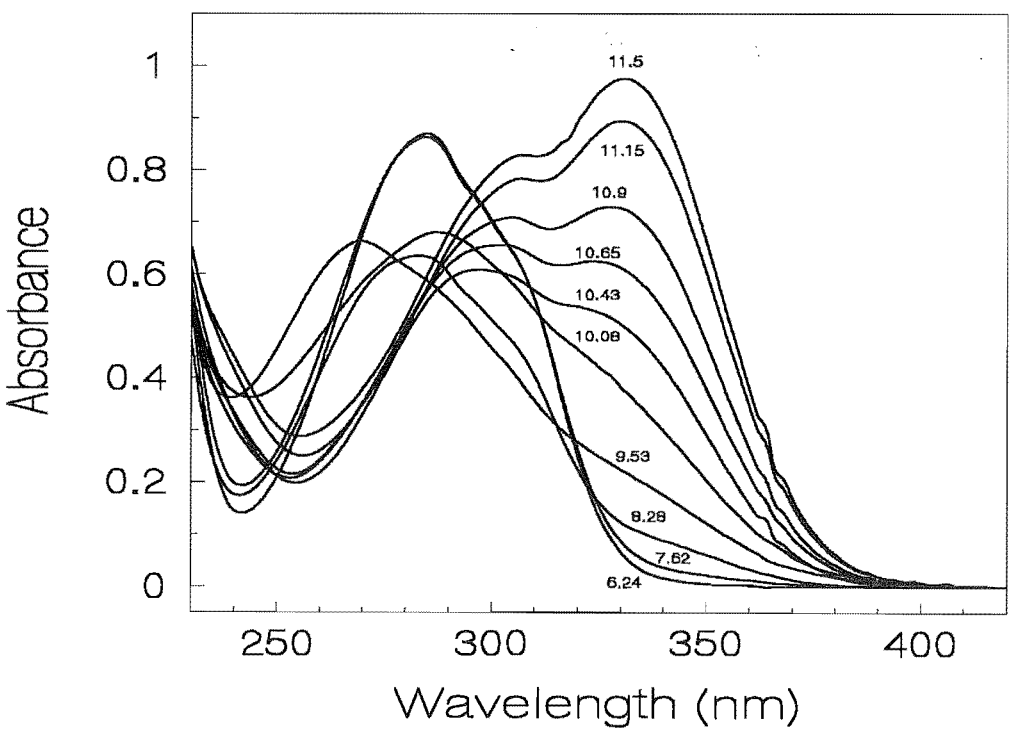


Figure 5.9 Effect of pH on the absorbance spectrum of *p*-coumaric acid.

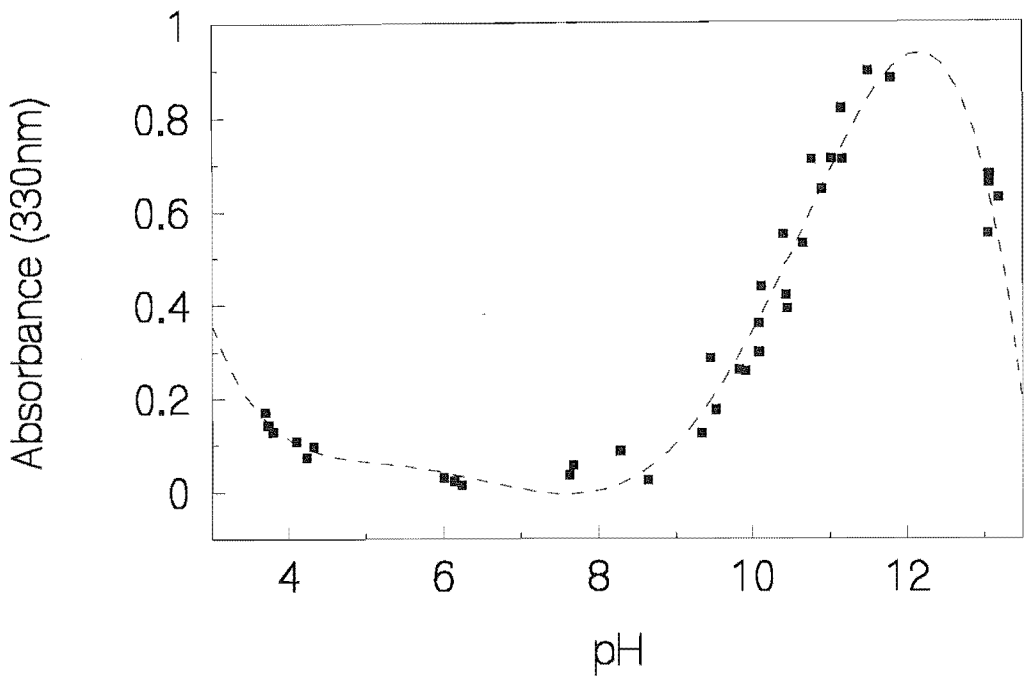


Figure 5.10 Effect of pH on the absorbance at 330nm of *p*-coumaric acid.

5.4 Results

Tubers of field grown plants were not usually exposed to any light, and yet some cultivars produce large quantities of anthocyanin. The absence of light reaching field grown tubers in the soil was confirmed with a light meter. This showed that no light penetrated through the soil beyond a depth of 2.5mm (Table 5.3), whereas tubers were usually found at a depth of 10-25cm, depending upon the cultivar.

Table 5.3 Depth of sunlight penetration through soil.

Depth (mm)	Light intensity (Watts/m^2)	% of sunlight
0	475	100.0
0.5	80	16.8
1	8.1	1.71
2	0.5	0.11
2.5 and greater	0	0

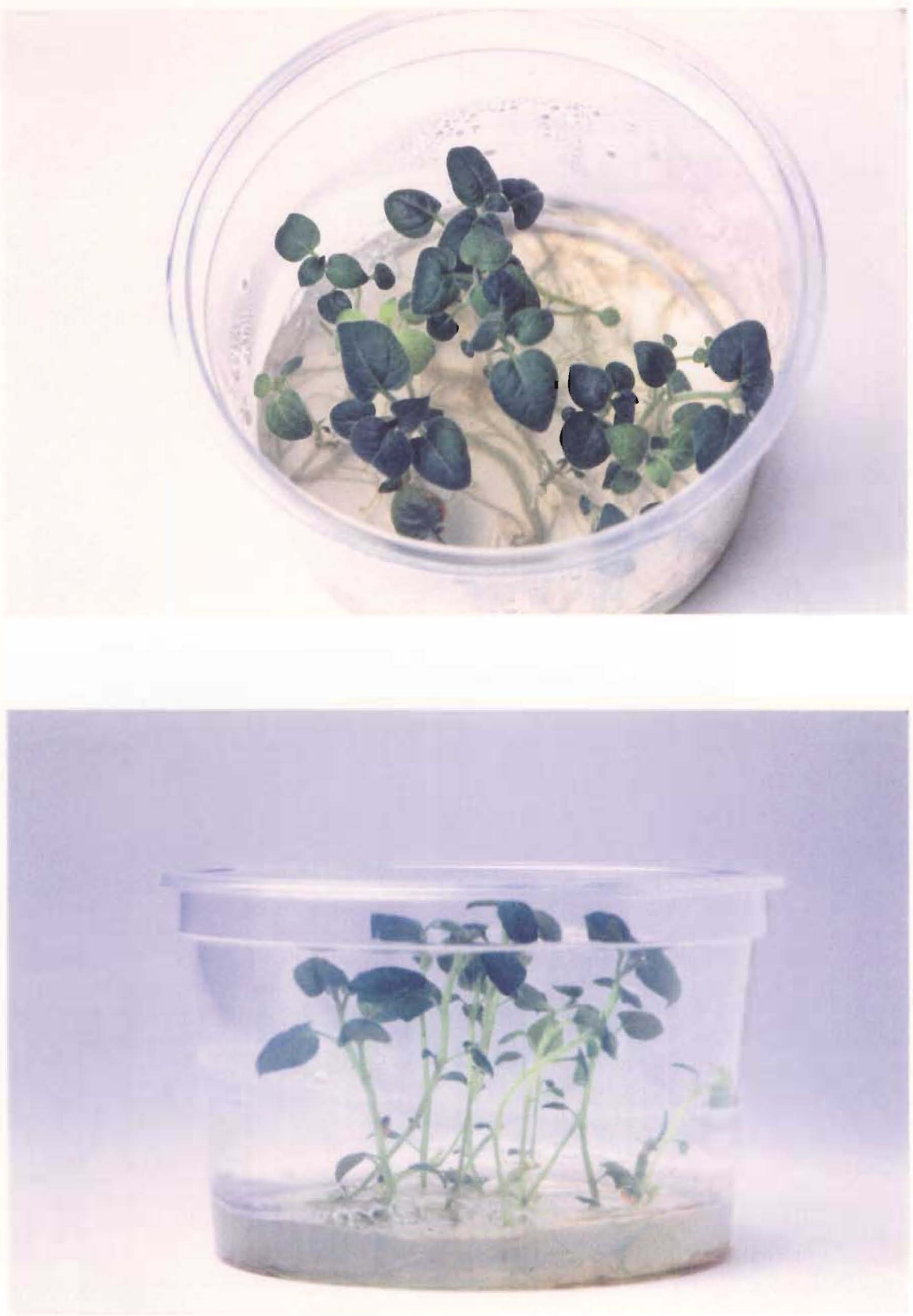


Figure 5.11 Tissue-cultured Desirée potato plantlets grown in the light (LG) for two weeks.

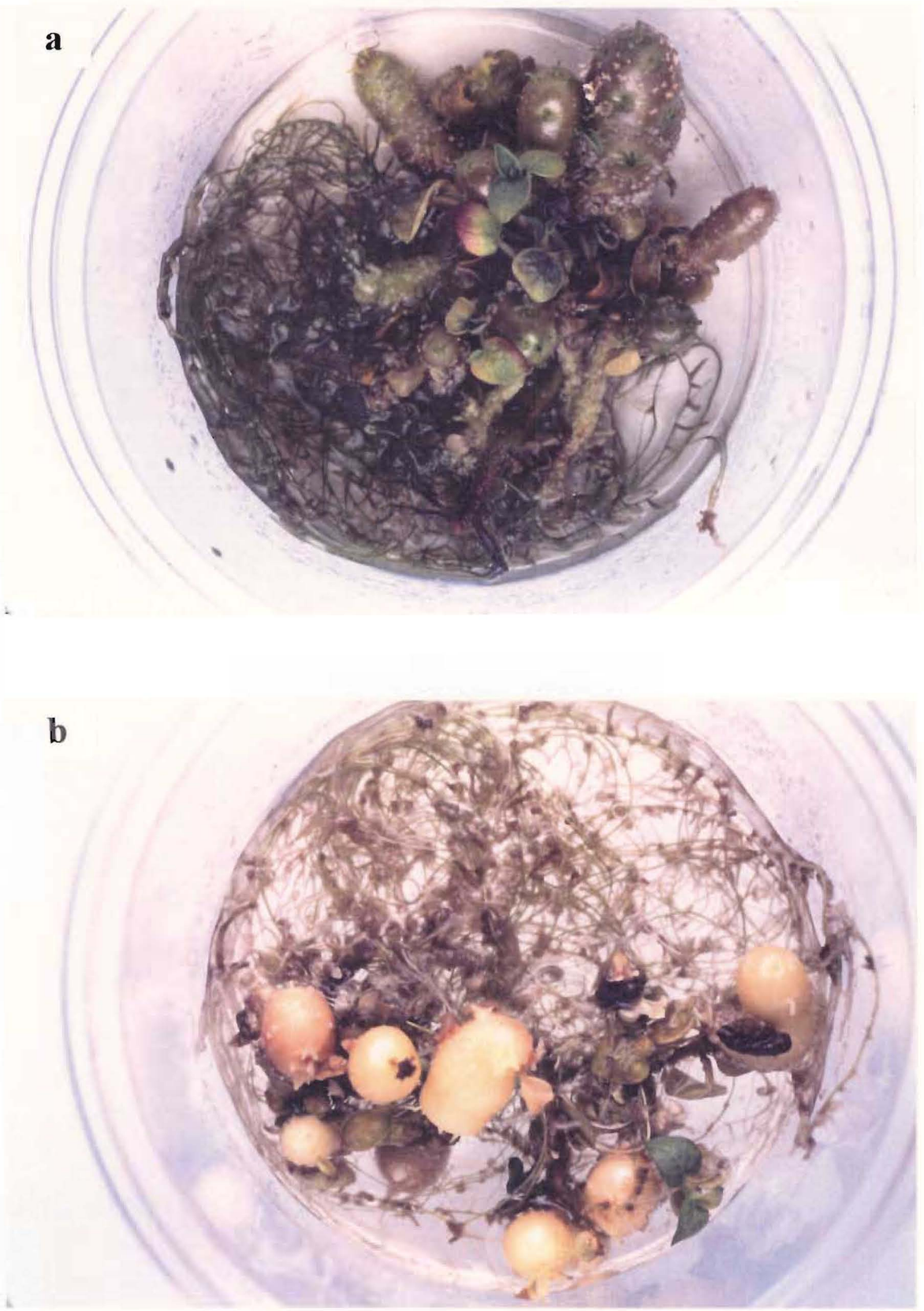


Figure 5.12 Tissue-cultured Desirée plantlets grown in the light for two weeks, followed by minituber production a) in the light (LG/LT) and b) in the dark (LG/DT) for nine weeks.

Initially, the effect of light on anthocyanin biosynthesis was tested using mature field grown Desirée tubers placed in a growth room under continuous light (150Watts/m^2) at 22°C. Anthocyanin concentration in skin samples increased slightly over a fourteen day period, however this may have been caused by the loss of water, because remaining tubers were extremely shrivelled. Also, the increased anthocyanin concentration was not statistically significant because there was a high variation among tubers. For these reasons and those already discussed (Section 5.1.5), minitubers were used to study further the effects of light on flavonoid biosynthesis.

5.4.1 Effect of light on the growth of minitubers

Desirée plantlets were grown in the light (LG) (Figure 5.11) and dark (DG), and then placed into TIM (tuber initiation media) and tubers grown in the light (LT) (Figure 5.12a) or dark (DT) (Figure 5.12b) for nine weeks. Additionally, light-grown plantlets were placed in TIM in the dark for two weeks and then in the light for the remaining seven weeks (LG/D2weeks/LT), because Hussey and Stacey (1984) found that darkness was best for *in vitro* tuberization. This was confirmed in our experiments with a 17% increase in the number of minitubers produced. However, there was a 43% decrease in tuber weight, giving a total yield per plant of only 67% of the full light treatment (Table 5.4), so this treatment of a two week dark incubation was not used in further experiments. The best treatment for optimal minituber production (weight per plant) was when both plantlets and tubers were grown in the light (LG/LT) (Table 5.4).

Table 5.4 Number and weight of minitubers produced by different light treatments (FW = fresh weight).

Treatment	Number of plants	Number of tubers/plant	FW per tuber (mg)	Tuber FW per plant (mg)
LG/LT	80	1.11	470	521.7
DG/LT	64	0.56	439	245.8
LG/DT	69	0.81	271	219.5
DG/DT	91	0.30	156	46.8
DG/DG/DT	45	0.07	78	5.5
LG/D2weeeks/LT	85	1.30	268	348.4

In general, plantlets grown in the light produced a higher number of tubers, whereas the tubers produced in the light had larger tubers, irrespective of whether the plantlets were grown in the light or dark (Table 5.4). Plantlets grown in the dark for two cycles (*i.e.* plantlets were grown in the dark for two weeks, subcultured and grown in the dark for another two weeks) had long thin stems with no, or only rudimentary leaves, and produced only the occasional very small minituber. However, if these plants were placed in the light they were still viable, and after subculturing in the light, produced normal plantlets.

5.4.2 Effect of nodal position on minituber production

The effect of the nodal position from which the plantlet originated on the production of minitubers was examined using Desirée plantlets. The original plantlets were cut into three portions; the top 5-8mm, including one pair of fully expanded leaves and the apical meristem (N1), the second pair of fully expanded leaves from the top (N2), and the third pair of leaves from the top (N3). These nodal explants were grown separately in the LG/LT treatment as before, and the number, weight and anthocyanin concentration of the resulting minitubers was measured. The position of the node from which the plantlet was derived affected the number and weight of tubers produced, with plantlets from the top nodes having fewer, but larger tubers than those from lower nodes (Table 5.5). There was a small difference in anthocyanin concentration, and tubers from higher nodes contained higher concentrations of anthocyanin than those from lower nodes, but this difference was not significant between adjacent nodes, although there was a significant difference between anthocyanin concentrations of N1 and N3 (Table 5.5). Because of these differences, only the top two nodes were used to grow plantlets for transfer into TIM in all further experiments.

Table 5.5 Effect of nodal position on minituber and anthocyanin production. (a, b = averages with the same letter in a column are not significantly different $p < 0.05$ calculated using Duncan's (SNK) test) (FW = fresh weight).

Node	Number of tubers/plant	FW per tuber (mg)	Tuber FW per plant (mg)	Anthocyanin (ng/cm^2SA)	Number of tubers used
N1	1.09^a	463^a	504^a	2.58^a	37
N2	1.13^a	388^b	438^b	$2.39^{a,b}$	44
N3	1.35^b	266^c	539^a	2.16^b	54

5.4.3 Effect of light on flavonoid biosynthesis

5.4.3.1 Desirée

The effect of growing Desirée plantlets and minitubers in the light or dark, on the concentrations of anthocyanins, flavonoids and phenolic acids is shown in Table 5.6. Light grown minitubers (LT) contained twice the concentration of phenolic acids of dark grown minitubers (DT). There was no difference in total phenolic concentrations between LG/LT and DG/LT nor between LG/DT and DG/DT treatments, therefore it appears that the total phenolic concentration was determined by the light conditions when the minitubers were forming and was not influenced at all by whether the plantlet was grown in the light or dark (Table 5.6). Light grown tubers (LT) contained five times the concentration of flavonoids of dark grown tubers (DT) and contained over 3ng/cm^{2}SA anthocyanin, whereas in dark grown Desirée tubers, no anthocyanin was detected by diode-array spectrophotometry or by HPLC.

Table 5.6 Phenolic concentrations in light and dark treated Desirée minitubers (se = standard error, SA = surface area).

Treatment	Anthocyanin ng/cm^{2}SA	se	Flavonoid µg/cm^{2}SA (µg/gFW)	se	Phenolic acid µg/cm^{2}SA (µg/gFW)	se
LG/LT	3.238	0.191	4.869	0.667	86.90	3.49
	(437.8)		(100.9)	(5.0)	(1618.2)	(172.4)
DG/LT	3.552	0.310	5.187	0.225	90.31	4.06
	(479.5)		(106.8)	(6.9)	(1763.8)	(124.1)
LG/DT	0	0	0.892	0.101	48.76	7.61
			(20.2)	(1.8)	(929.3)	(41.9)
DG/DT	0	0	1.006	0.209	40.48	5.97
			(24.3)	(5.0)	(987.6)	(136.1)

The results for anthocyanin concentrations were presented per cm^2 of surface area (SA), because anthocyanins were found only in the skin, with none detected in the flesh, and the weight of the skin could not be determined. (Surface area was also used for experiments carried out in Chapter 4, see Section 4.1.5). Flavonoids and phenolic acids may have been over estimated by the calculation of concentrations per cm^{2}SA because these compounds occurred in the flesh as well as in the skin, although phenolic acids in the flesh occurred only at 10% of the level as in the skin, and flavonoids at less than 5%.

However, it is thought that this over estimation would be minimal, because the concentrations of flavonoids and phenolic acids in the flesh were low, particularly in the small minitubers with a large surface area to volume ratio. Flavonoids and phenolic acids calculated per gFW showed the same pattern as when calculated per cm^2SA (Table 5.6), so results were presented per cm^2SA for flavonoids and phenolic acids as well as anthocyanins in all further experiments, so concentrations could be compared directly for all three phenolic classes. However, flavonoid and phenolic acid concentrations could only be directly compared between treatments with similar sized minitubers because the proportion of skin (SA) to flesh (volume) varied in different sized tubers.

5.4.3.2 Other cultivars

Similar experiments to those with Desirée plantlets (Section 5.4.3.1) were carried out with tissue-cultured plantlets of Amadeus, Bildtstar, RKE and Urenika to get a range of anthocyanin colours and intensities. Amadeus and Bildtstar tubers were a similar colour and intensity to Desirée, whereas RKE tubers were a dark red colour and Urenika tubers were a dark purple/black colour (Figure 5.13).

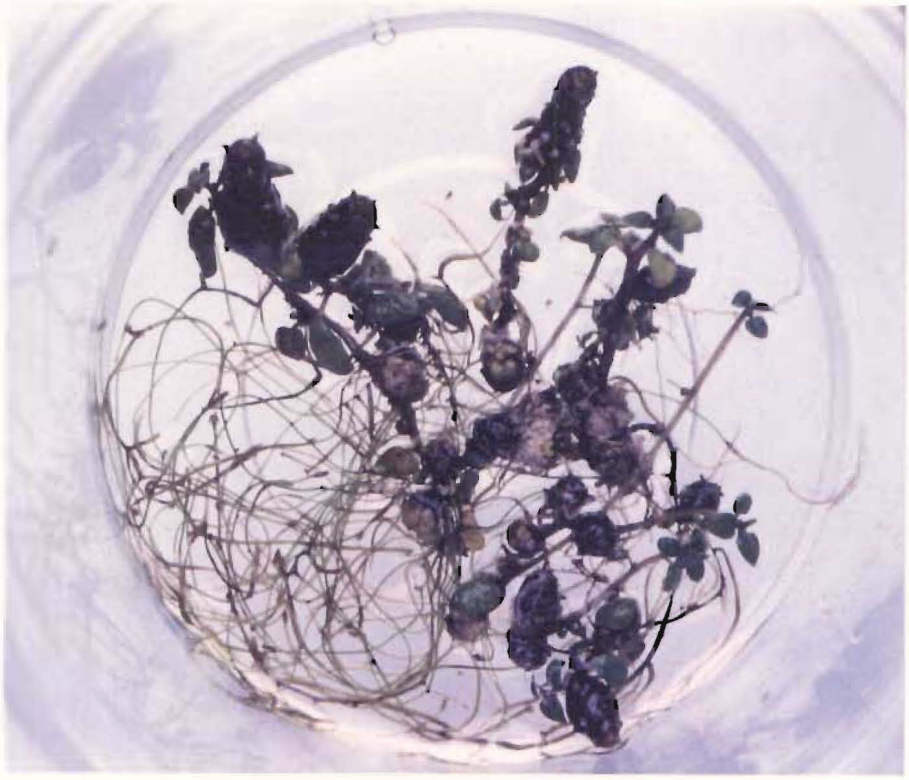


Figure 5.13 Tissue-cultured Urenika potato plantlets grown in the light in PMM for two weeks and TIM for nine weeks.

All minitubers contained anthocyanin only in the skin, and these cultivars showed similar patterns to Desirée minitubers, with anthocyanin concentrations higher in light treatments than dark treatments (Table 5.7). However, whereas Desirée minitubers produced in the dark (DT) contained no detectable anthocyanin, these other cultivars contained some anthocyanin, in varying quantities, except for Bildtstar in the dark (DG/DT) treatment which did not contain any anthocyanin. Tubers with a high anthocyanin concentration in field grown tubers also contained relatively high concentrations in minitubers grown in the dark, whilst tubers with low anthocyanin concentrations in field grown tubers contained little or no anthocyanin in minitubers grown in the dark (Table 5.7). Therefore, Amadeus, Bildtstar and Desirée produced none, to low concentrations of anthocyanin in both the light and dark, whilst RKE and Urenika produced higher concentrations at both treatments.

Table 5.7 Anthocyanin production in minitubers of different cultivars in the light and dark (se = standard error, SA = surface area, RKE = Red King Edward).

Cultivar	Anthocyanin concentration (ng/cm^{2}SA)					
	DG/DT	se	LG/DT	se	LG/LT	se
Amadeus	0.31	0.02	0.25	0.08	3.57	0.16
Bildtstar	0	0	0.13	0.04	2.62	0.12
Desirée	0	0	0	0	3.23	0.19
RKE	1.47	0.43	1.43	0.11	10.46	0.25
Urenika	9.97	1.15	11.02	0.82	27.32	1.31

5.4.4 Effect of differing light intensities on the biosynthesis of phenolics

Desirée plantlets were grown for two weeks in light boxes with varying amounts of shade cloth (*i.e.* different light intensities), then transferred to TIM and kept in the same light boxes (same shade conditions and light intensities) for nine weeks before analysis of minitubers. Anthocyanin concentration showed a curvilinear increase (second order polynomial regression $R^2 = 98\%$) from 0 to 3.2ng/cm^{2}SA with increasing light intensities (Figure 5.14a). The increase in the flavonoid concentration was more linear (second order polynomial regression $R^2 = 98\%$) and showed a four fold increase with increasing light intensities (Figure 5.14b). The phenolic acid concentration doubled, and showed a similar curve to that shown by the flavonoid concentration (second order polynomial regression $R^2 = 96\%$) (Figure 5.14c), with increasing light intensities.

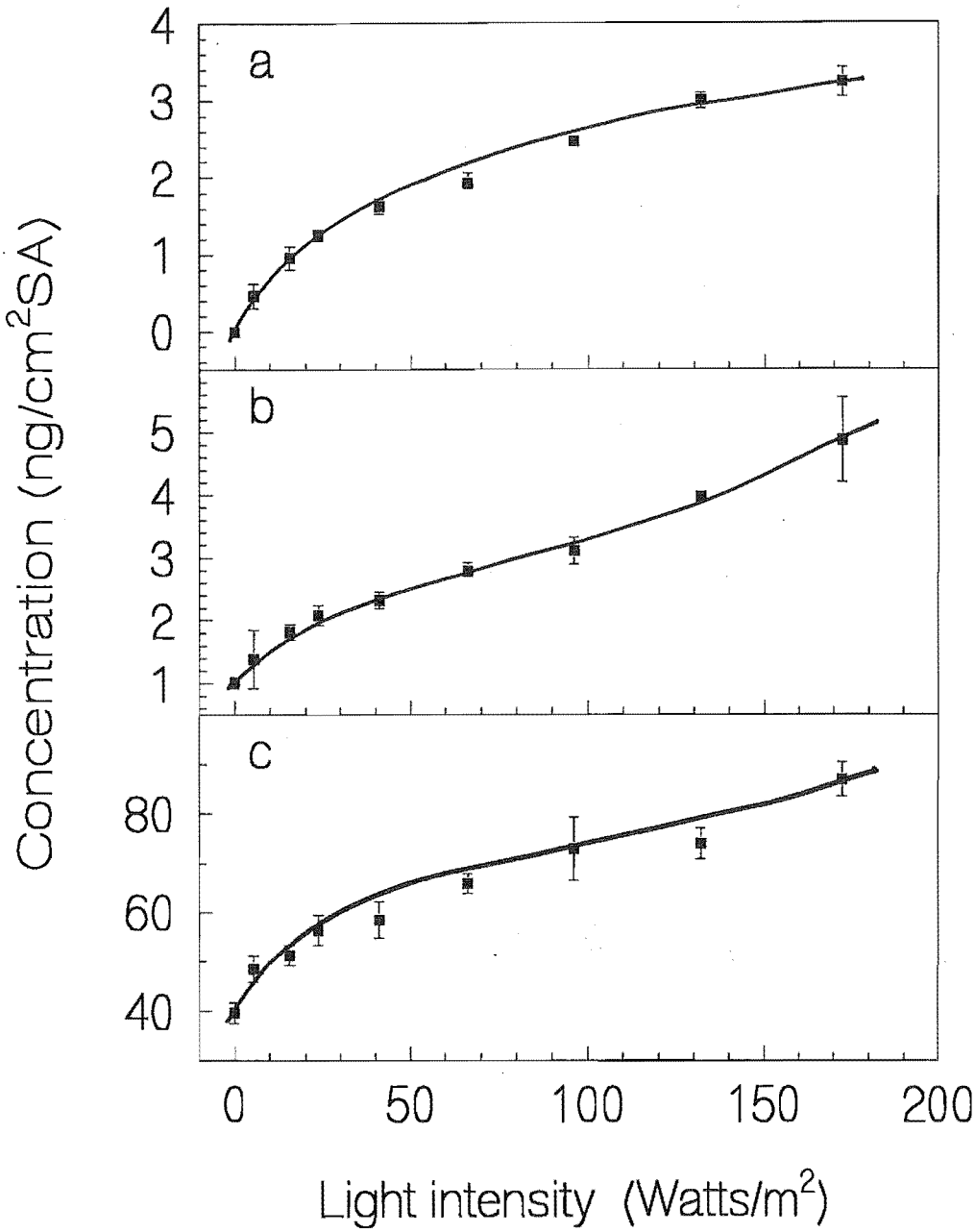


Figure 5.14 Effect of light on the concentrations of a) anthocyanins, b) flavonoids and c) phenolic acids. Error bars represent ± 1 standard error.

5.4.5 Effect of light at different wavelengths on anthocyanin biosynthesis

Desirée plantlets were grown in light boxes, as before (Section 5.4.4), except that coloured filters (or lamps for UV and far-red) were used to provide UV, purple, blue, yellow, red, far red, dark and white light treatments. Plants grown in the dark, UV, red

and far red light were etiolated and produced small minitubers, whilst plants grown in the other treatments looked similar to plants grown in the light. The effect of these light treatments on anthocyanin concentration is shown in Figure 5.15, with the anthocyanin concentration increasing with increasing wavelength, up to yellow light with the highest concentration of anthocyanin, before decreasing at higher wavelengths. However, it was found that these differences were not due entirely to the different wavelengths of light, because the total amount of light that these filters allowed into the light boxes varied considerably. Figure 5.16 shows the anthocyanin production from the minitubers grown under different wavelengths of light, compared with the anthocyanin production at different light intensities due to reduced light under shade cloth (Figure 5.14a). Purple, and especially blue and red light increased production of anthocyanin in minitubers compared with white light of the same intensity, whereas anthocyanin production in yellow light was the same as in white light of the same intensity. Although it was not carried out, it would also be interesting to compare anthocyanin production at the same intensity for each wavelength.

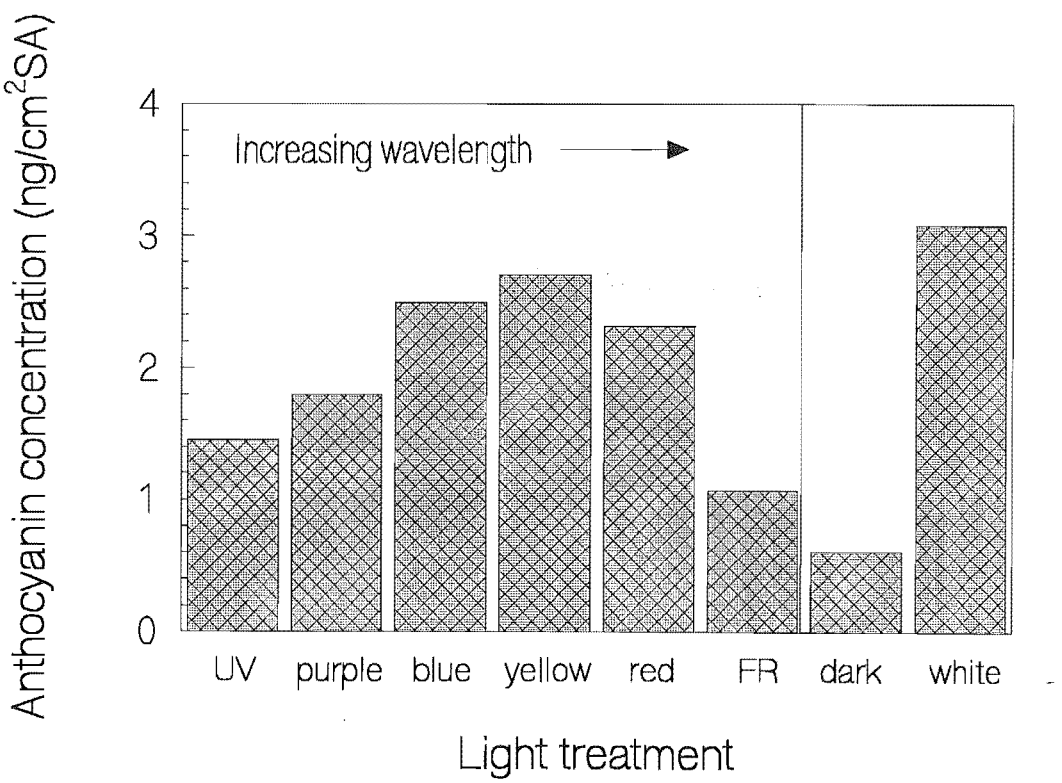
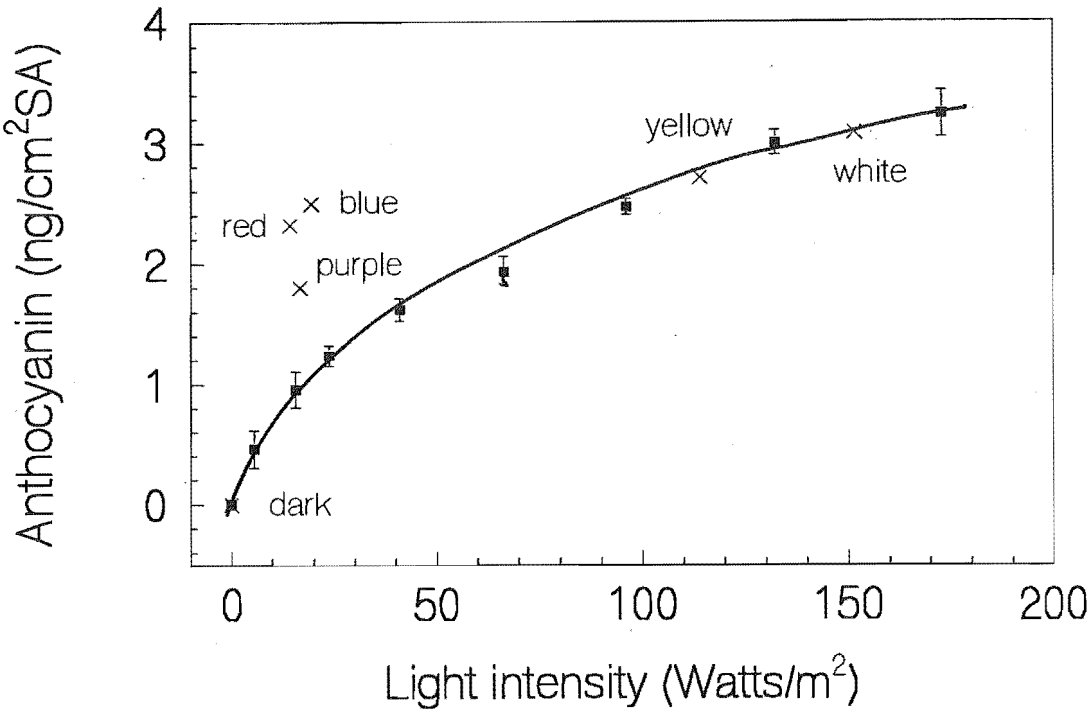


Figure 5.15 Effect of coloured light filters on anthocyanin concentration in Desirée minitubers.



■ Anthocyanin concentration of minitubers grown in white light at various light intensities (from Figure 5.14)

x Anthocyanin concentration of minitubers grown at different wavelengths of light (from Figure 5.15).

Figure 5.16 Increased anthocyanin production in minitubers grown in red, blue and purple light. Error bars represent ± 1 standard error.

5.4.6 Effect of length of light exposure on anthocyanin biosynthesis

Minitubers of Desirée were grown in a LG/DT treatment and, after nine weeks in the dark, exposed to light for varying lengths of time from 5min to 2 weeks, then the tubers stored in the dark for the remainder of the two week period, before analysis of anthocyanin concentrations. The anthocyanin concentration appeared to be linearly related to the length of exposure time of the minitubers to light, with increasing anthocyanin concentrations at increased exposure times (Figure 5.17, inset). However, when time was plotted on a logarithmic scale to expand the lower values (5min to 24h), it was found that a significant increase in anthocyanin concentration occurred only after 8h of exposure to light (Figure 5.17).

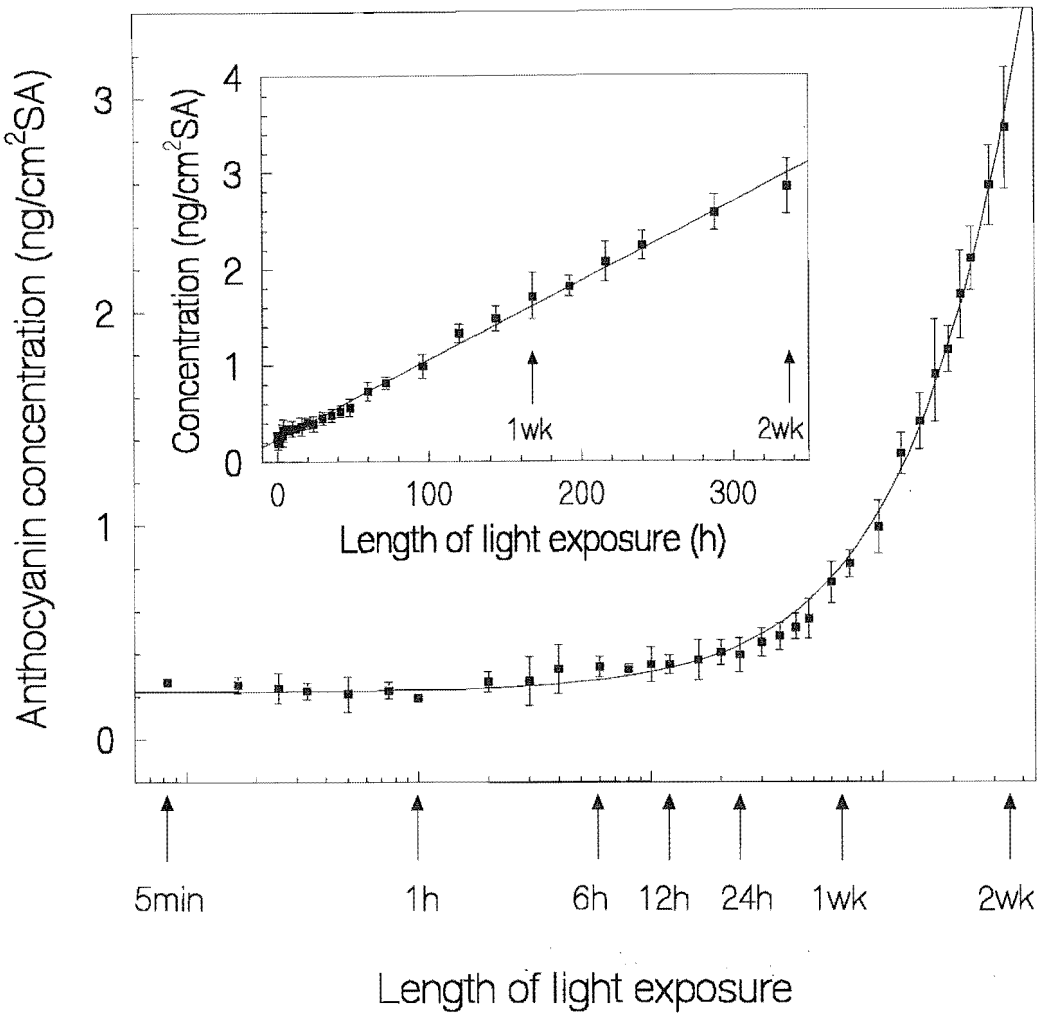


Figure 5.17 Effect of the length of light exposure on anthocyanin concentration. Tubers were grown in the dark (LG/DT) before being exposed to light for varying lengths of time and then stored in the dark for the remainder of two weeks. Error bars represent ± 1 standard error.

5.4.7 Effect of light on enzyme activities and biosynthesis of phenolics

To determine the effect of light on anthocyanin, flavonoid and phenolic acid biosynthesis and enzyme activities, Desirée minitubers were grown in the LG/DT treatment. After nine weeks the pottles were placed in the light, and samples of 20-30 minitubers were taken at intervals over up to four weeks for assay of both phenolic concentrations and enzyme activities. Anthocyanin concentration increased linearly up to about ten days,

and then remained at the same level of about 3ng/cm^{2}SA (Figure 5.18a). Flavonoid concentrations and phenolic acid concentrations also increased linearly, but had reached a maximum around four to five days (Figure 5.18b and c).

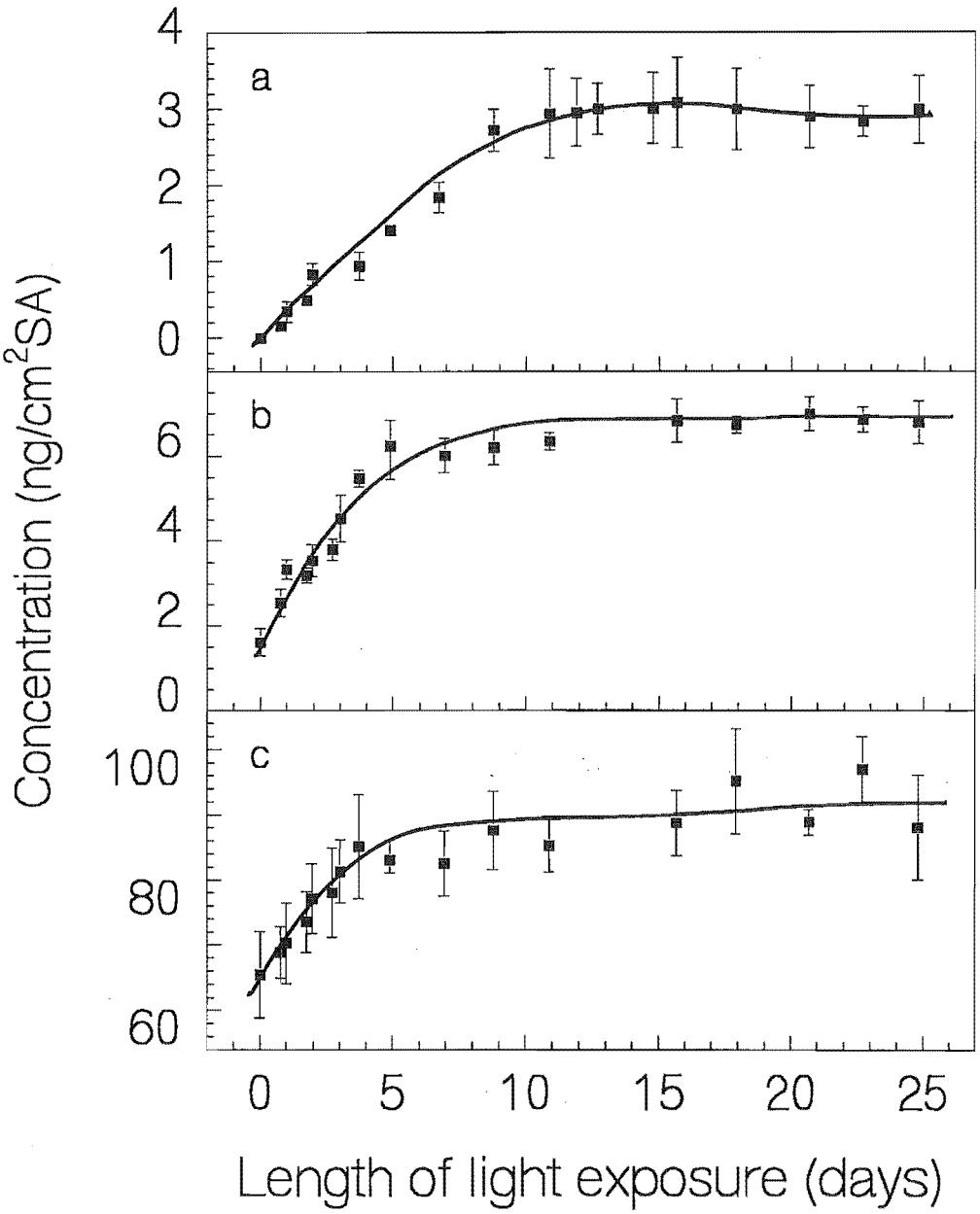


Figure 5.18 Effect of light exposure on concentrations of a) anthocyanins, b) flavonoids and c) phenolic acids. Minitubers were grown in the dark (LG/DT) for nine weeks before being exposed to light. Error bars represent ± 1 standard error.

PAL activity increased sharply to a maximum twenty hours after exposure to light and then decreased slowly (Figure 5.19), whilst CHI activity initially had no detectable activity before exposure to light, but subsequently increased to a maximum at about eleven days, before decreasing to zero again at twenty five days after the onset of light exposure (Figure 5.19).

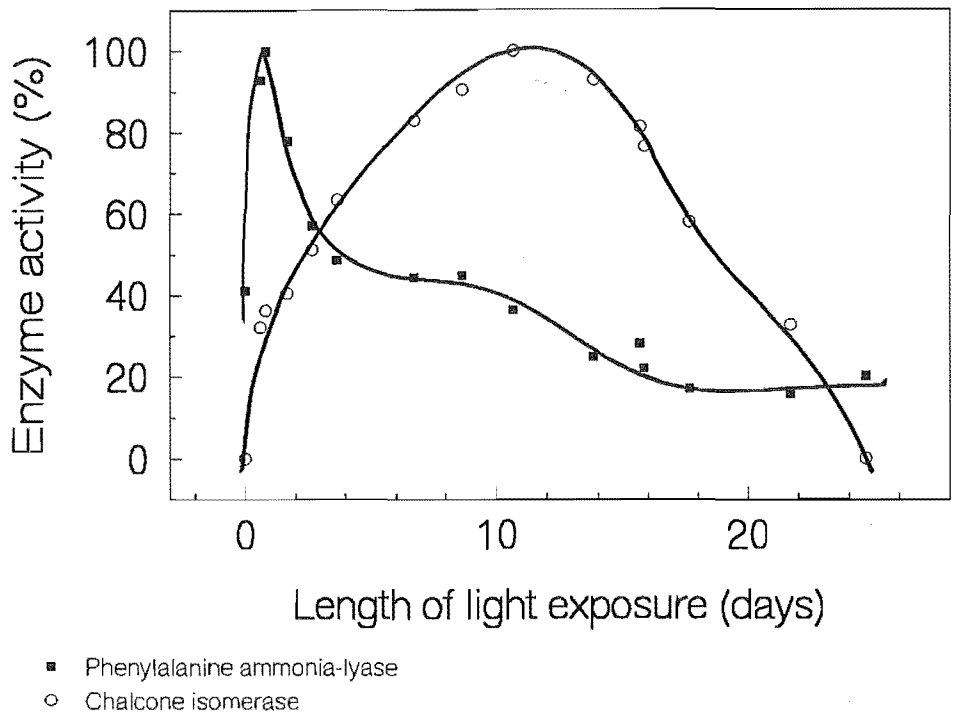


Figure 5.19 Phenylalanine ammonia-lyase (PAL) and chalcone isomerase (CHI) activities in minitubers following exposure to light. Minitubers were grown in the dark (LG/DT) for nine weeks before light exposure.

The other enzyme activities were only measured over a fifteen day period.

Both C4H and DHR showed no detectable activity before exposure to light, and then showed a small lag period, after which C4H increased to a maximum at about nine days and subsequently decreased, whereas DHR continued to increase over the two week period (Figure 5.20).

Initially F3H activities were low, but activities with both substrates (naringenin and eriodictyol) increased upon exposure to light, reaching a maximum at about nine days, before decreasing (Figure 5.21). Activity with eriodictyol was typically higher than that with naringenin, and at maximum activity for both substrates (nine days), activity with naringenin was only 38% of that with eriodictyol (Figure 5.21).

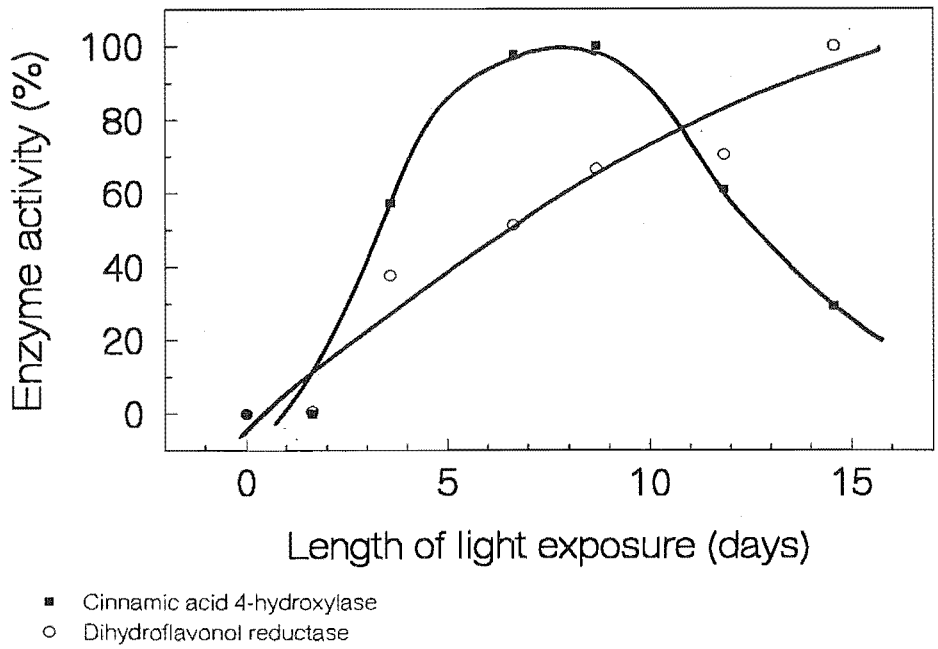


Figure 5.20 Cinnamic acid 4-hydroxylase (C4H) and dihydroflavonol reductase (DHR) activities in minitubers following exposure to light. Minitubers were grown in the dark (LG/DT) for nine weeks before light exposure.

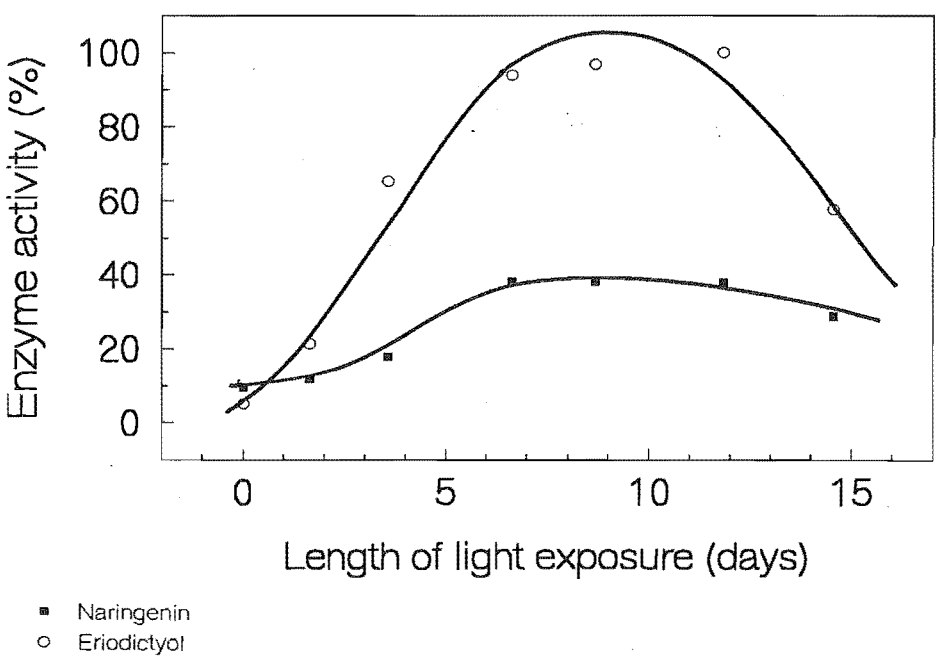


Figure 5.21 Flavanone 3-hydroxylase activity (F3H) (with naringenin or eriodictyol as substrates) in minitubers following exposure to light. Minitubers were grown in the dark (LG/DT) for nine weeks before light exposure.

Activities of F3'H were initially low, but then showed variable increases depending on substrate (Figure 5.22). After two weeks of light exposure, activity with naringenin and *p*-coumaric acid was high, whilst activity with Km was low, and even lower with apigenin (Figure 5.22).

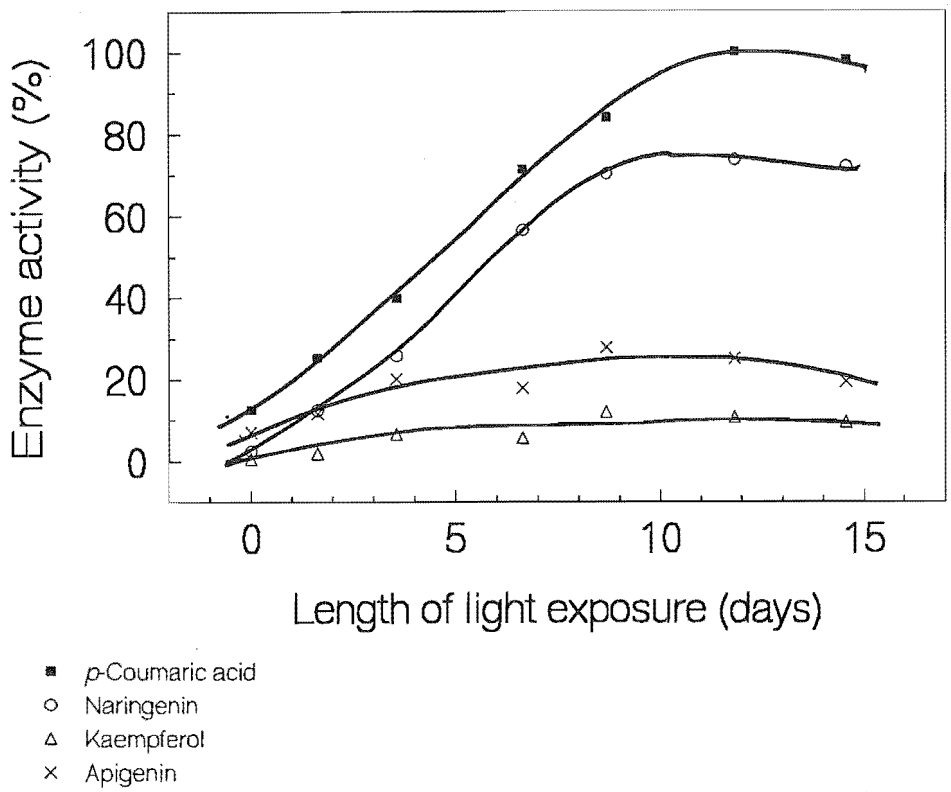


Figure 5.22 Flavonoid 3'-hydroxylase (F3'H) activity (with *p*-coumaric acid, naringenin, kaempferol (Km) or apigenin as substrates) in minitubers following exposure to light. Minitubers were grown in the dark (LG/DT) for nine weeks before light exposure.

GT activity increased after exposure to light, but not to the same extent as for other enzymes (Figure 5.23). High background levels of GT were found (before light exposure), especially with Km and UDP-glucose, and also Qu and UDP-glucose as substrates. UDP-glucose was the best sugar substrate, with UDP-galactose having between 40-80% of this activity, depending on the flavonoid substrate (Figure 5.23). The highest activity was found with Km and UDP-glucose as substrates, whereas activities with Qu and UDP-glucose, Km and UDP-galactose, and Qu and UDP-galactose as substrates were about 50% of the activity with Km and UDP-glucose. Luteolin showed low activity with both these sugars (Figure 5.23).

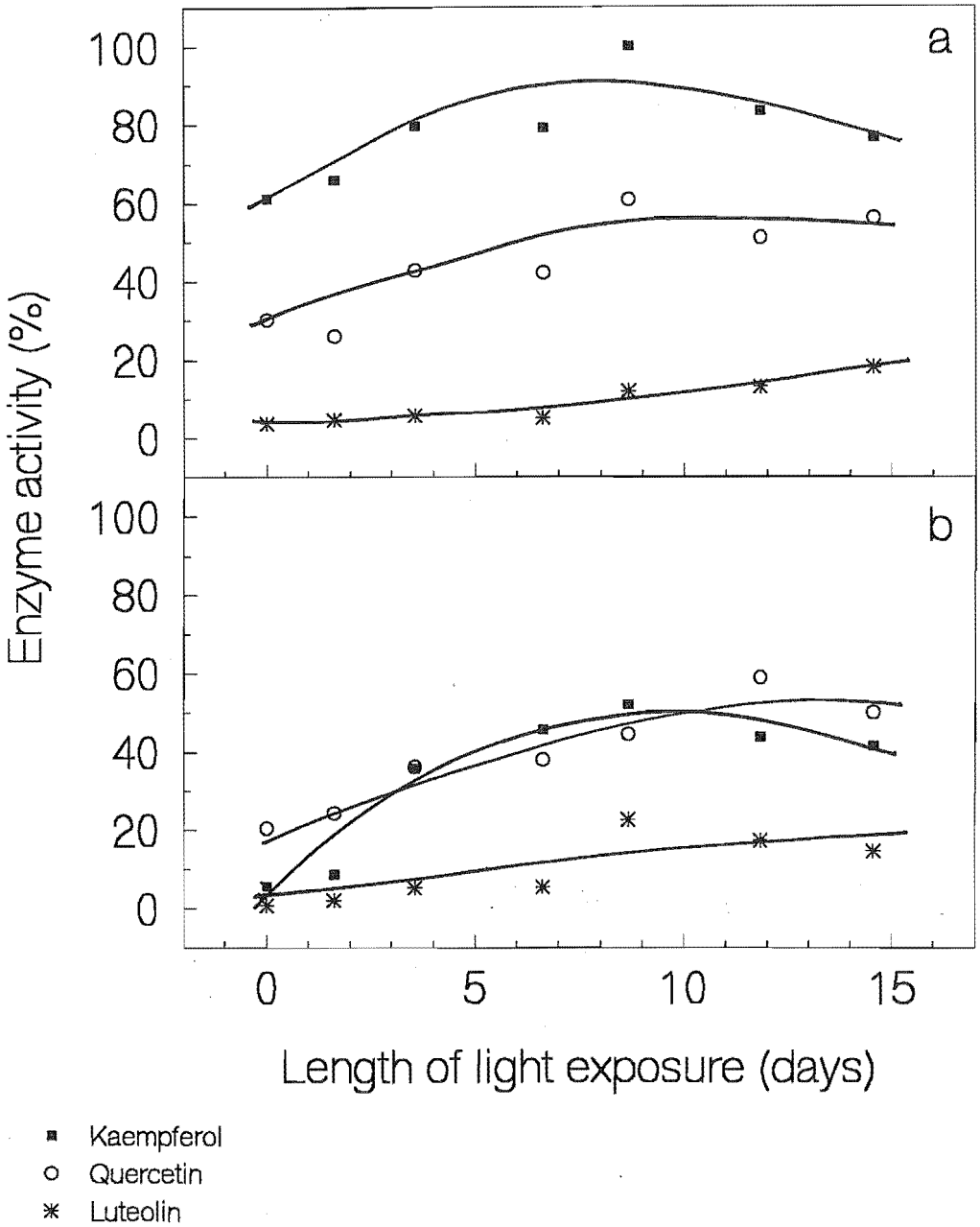


Figure 5.23 Glycosyltransferase (GT) activity in minitubers following exposure to light. a) UDP-glucose or b) UDP-galactose as the sugar substrates, each with kaempferol (Km), quercetin (Qu) or luteolin as the flavonoid substrates. Minitubers were grown in the dark (LG/DT) for nine weeks before light exposure.

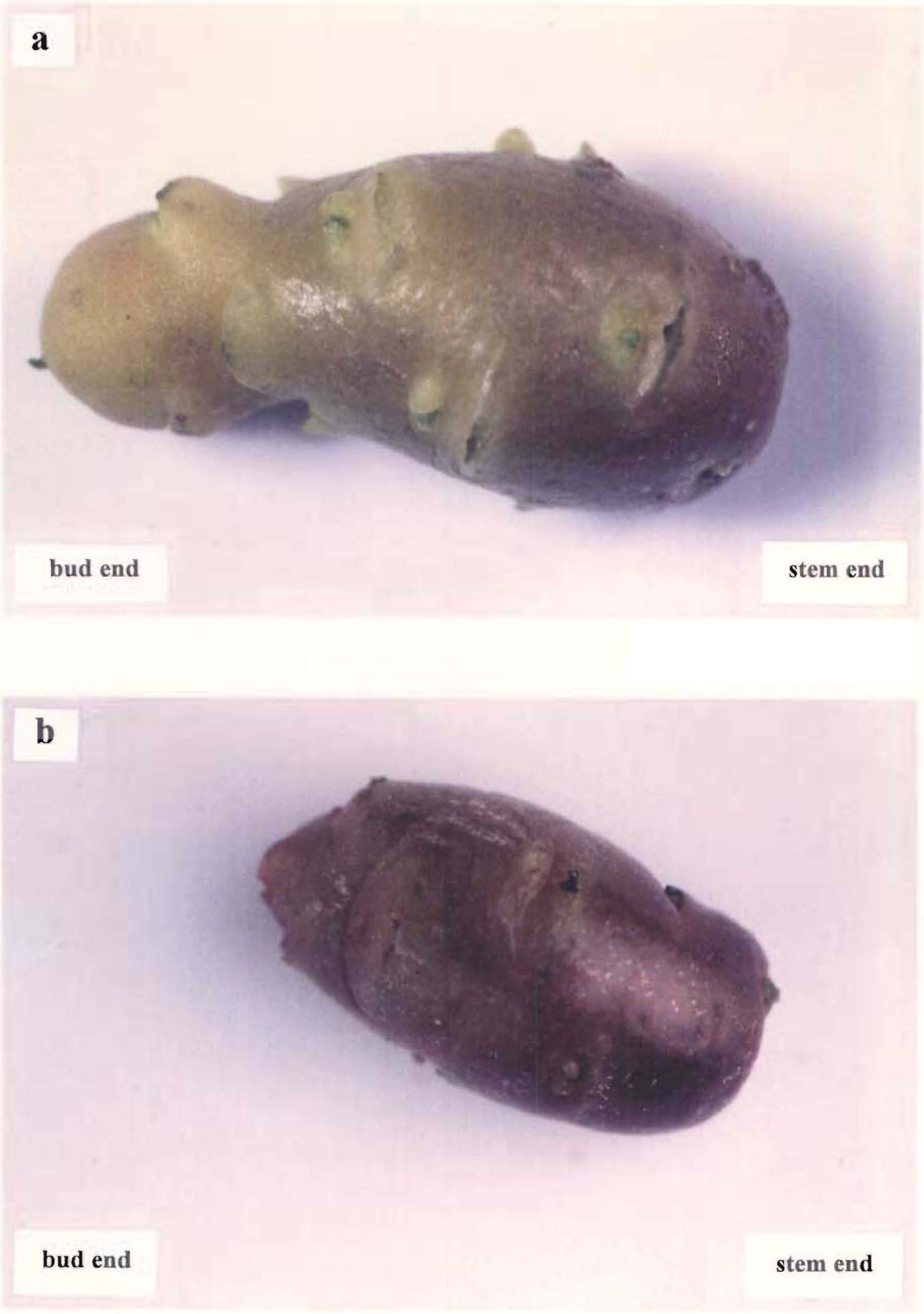


Figure 5.24 Examples of a) Desirée and b) Red King Edward (RKE) minitubers showing the development of anthocyanin colour from the stem end, after exposure to light

5.4.8 Distribution of anthocyanin within the minituber

Minitubers were grown in LG/DT treatment and then exposed to light as in Section 5.4.7. The anthocyanin began to form from the stem end first, then was gradually synthesised towards the bud end. This can be seen for examples of a Desirée (Figure 5.24a) and the more intensely coloured RKE minituber (Figure 5.24b). Additionally, experiments were carried out where the white Desirée minitubers, grown in the LG/DT treatment, were removed from the plantlets and incubated in the light. There was a much lower production of anthocyanin than in the tubers which remained attached to the plantlets, for the same time period. In unattached minitubers, the anthocyanin formed was visible mostly on the top side of the minituber (which was exposed to the light), with no apparent difference between the stem and bud end. Unfortunately, these minitubers began to dry out within a few days, because of the high surface area to volume ratio, so observations were limited to this short time period.

5.4.9 Covering minitubers with foil covers

This experiment was designed to simulate, as closely as possible, the field grown tuber light regime, with the tubers in the dark and the leaves in the light, to determine whether exposure of the leaves (but not the tubers) to light increased anthocyanin, flavonoid and phenolic acid biosynthesis. This was complicated by the fact that minitubers were often produced on the stems, amongst the leaves.

Minitubers from Desirée plantlets were grown up in the LG/DT treatment and then (after the LG/DT treatment) individual minitubers were wrapped in sterile aluminium foil, to exclude all light from reaching the minituber. On some plantlets, tubers were left uncovered as controls. Plantlets were incubated in the light for three weeks, and the concentrations of anthocyanins, flavonoids and phenolic acids in minitubers measured before light incubation (time 0), and after two and three weeks of light incubation.

Before light incubation, no anthocyanins were found in the minitubers (Figure 5.25a). After two weeks of light exposure the anthocyanin concentration in the light exposed minitubers had reached 3.5ng/cm^{2}SA and there was no further increase found in the light exposed tubers by three weeks (Figure 5.25a). Therefore, by two weeks of light incubation the anthocyanin concentration had reached a maximum, as was found in light exposed minitubers described in Section 5.4.7. In the foil covered minitubers, after exposing the plant to light for two weeks, the anthocyanin concentration had increased from no anthocyanin to 40% of this maximum level (of light exposed minitubers). By

three weeks, the anthocyanin concentration in the covered minitubers had reached almost 90% of the maximum found in light exposed minitubers (Figure 5.25a).

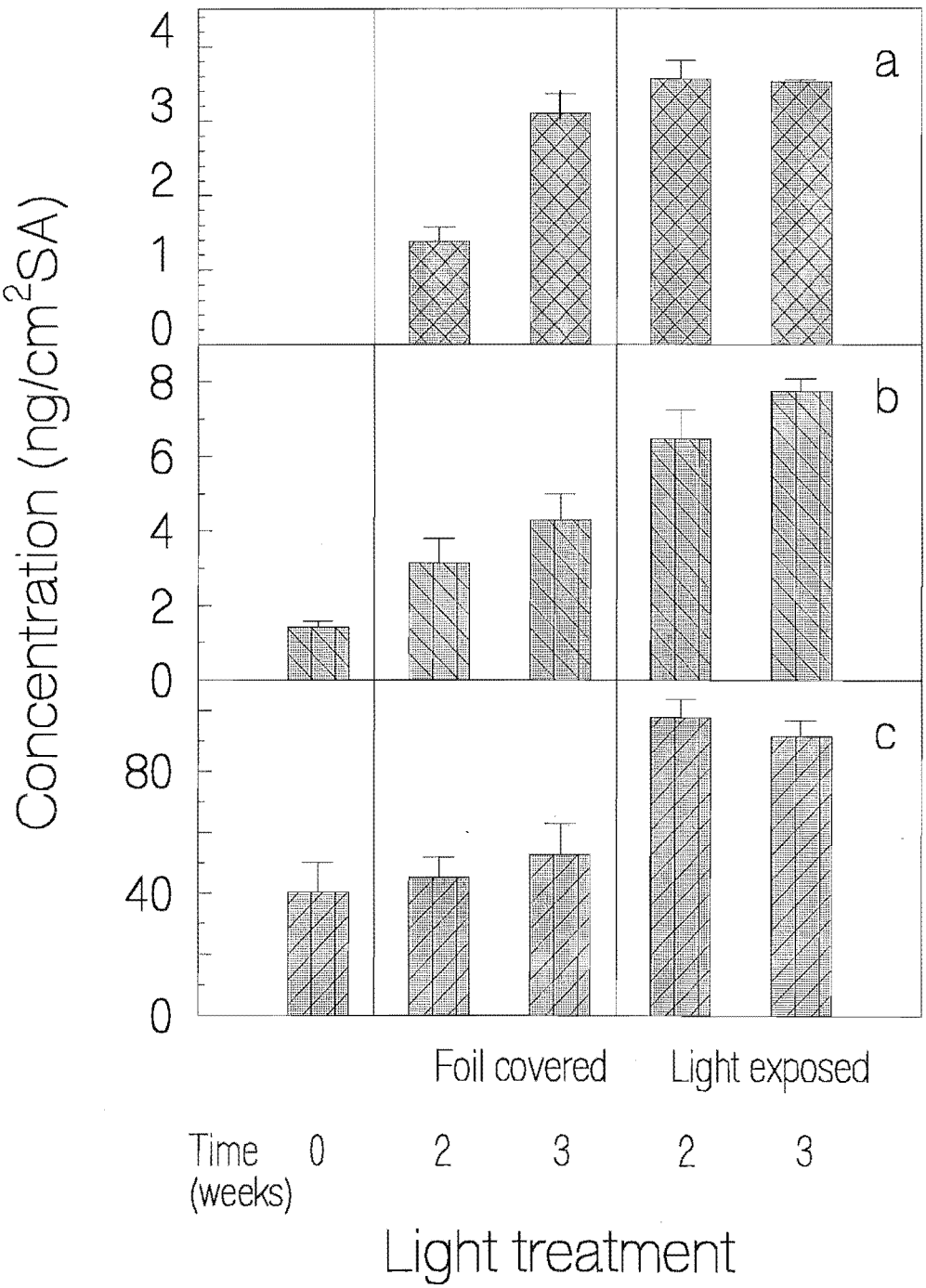


Figure 5.25 Concentration of a) anthocyanins, b) flavonoids and c) phenolic acids found in dark grown minitubers, before (time = 0) and after exposure to light (2 and 3 weeks) with the minitubers covered or not covered with foil. Error bars represent ± 1 standard error.

Flavonoids were found at low concentrations (1.5ng/cm^{2}SA) in the minitubers before light exposure (Figure 5.25b). After light exposure, the flavonoid concentration in the light exposed tubers had increased over four times to 6.3ng/cm^{2}SA, and by three weeks, increased further to 7.7ngcm^2/SA (Figure 5.25b). In the covered minitubers the flavonoid concentration doubled in the first two weeks, to 3.1ng/cm^{2}SA, and then increased again in the next week to 4.3ng/cm^{2}SA, three times the initial (time 0) concentration (Figure 5.25b). However, after three weeks the flavonoid concentration of the foil covered minitubers after light exposure was still less than 60% of the flavonoid concentration in light exposed minitubers.

The phenolic acid concentration of minitubers before light exposure was 41ng/cm^{2}SA, which increased to about 95ng/cm^{2}SA after light exposure (two or three weeks) (Figure 5.25c). Although there appeared to be a small increase in the concentration of phenolic acids in foil covered minitubers after exposure of the plantlet to light (from 40ng/cm^{2}SA to 52ng/cm^{2}SA), this increase was not significant (Figure 5.25c).

5.4.10 Grafted potatoes

Grafts were carried out with potato plants grown in plastic bags in glass houses at the NZ Institute for Crop & Food Research Ltd, Lincoln, and with the assistance of Ms Jill Reader. Scions (photosynthetic portions) from cultivars with red/pink tubers were grafted onto rootstocks from cultivars with white tubers, to determine whether the tops from cultivars with coloured tubers had any control over the colour of white tubers, and *vice versa*. 'Rootstocks' included the fibrous root system, and may have also included some rhizomes, although grafting was carried out before tubers were initiated. For each graft, the bag and complete rootstock tissue to above the graft union was covered with aluminium foil to prevent any light from reaching the rootstock. This was to ensure that all photosynthetic products and any other light induced signals (e.g. phytohormones) to the root stock tubers came from the scion. The grafts are listed in Table 5.8.

Table 5.8 Grafts carried out between red and white potato plants.

Rootstock	Colour	Scion	Colour	Resultant tuber colour
Ilam Hardy	white	Desirée	pink/red	white
Iwa	white	Desirée	pink/red	white
Rua	white	Desirée	pink/red	white
Rua	white	Red Rascal	pink/red	white
Iwa	white	Red Rascal	pink/red	white
Desirée	pink/red	Ilam Hardy	white	pink/red

Tubers were harvested 56 days after grafting. Anthocyanins were extracted separately from every tuber from each plant, by taking approximately 2g of skin tissue from each tuber. Extracts were analysed by diode-array spectrophotometry and HPLC. Tubers from white rootstocks contained no detectable anthocyanins, whereas the tubers from the Desirée rootstock showed no change from typical Desirée anthocyanin concentrations (Table 5.8). Therefore, the anthocyanin colour appeared to be determined by the rootstock, and grafting a red top onto a white rootstock had no effect on the anthocyanin production in the tubers of any of the cultivars with white tubers.

5.5 Discussion

5.5.1 Techniques

A number of methods were investigated and optimised for the analysis of enzyme activities. Although attempts were made to use simple spectrophotometric assays for most of these enzymes, this was only possible for PAL and CHI. The other enzymes were all assayed by analytical HPLC. This had a greater sensitivity and enabled the substrates and different products to be distinguished, and their identity confirmed to ensure the correct reaction was being measured. Although microsomal extracts were used for the assay of C4H and F3'H activities, some activity could also be detected in the crude acetone enzyme extract, but was at much lower levels. This confirms that C4H and F3'H are found in the membrane fraction, as previously reported (reviewed in Stafford 1990). Enzyme activities from minitubers tended to be quite low. It is hypothesised that this was because the whole tuber was used for enzyme extraction, whereas the phenolic products and enzyme activities were generally significantly higher in the skin (Chapter 3). Enzyme activities were calculated per mg protein, and therefore the inclusion of the flesh for the extracts would mean that all the other proteins (e.g. enzymes involved in carbohydrate metabolism, and storage proteins, such as patatin) would also have been extracted.

The measurement of the amount of light penetrating the soil showed that normal tubers grown in the field were not exposed to any direct light. Although the increase in anthocyanin was not significant in mature Desirée tubers exposed to light, Brown and Riley (1976) found that after exposure of mature tubers to light, only one of the 29 cultivars with "white" skinned tubers tested failed to develop anthocyanin pigmentation in the tuber skin. It has been shown that most flowers and fruits require light for anthocyanin biosynthesis, and in almost all plants which have been investigated, flavonoids accumulated rapidly after exposure to light (Hahlbrock *et al.*, 1976;

Grisebach, 1982; Mancinelli, 1985; Beggs *et al.*, 1986; 1987; McClure, 1986; Brödenfeldt and Mohr, 1988). So why do the underground tubers which develop in the dark produce anthocyanin?

Tissue-cultured minitubers were used as a tool to investigate the effects of light on anthocyanin, flavonoid and phenolic acid biosynthesis in potatoes. The use of such minitubers has a number of advantages over the use of field grown tubers. The main advantages were 1) that control of the environmental conditions was easier because of the small size of the plantlets, and minitubers attached to the growing plants were easily exposed to different light regimes and, 2) the variation between tubers was reduced. This was especially important because field grown tubers were found to be very variable in factors such as colour, phenolic concentrations and enzyme activities. Minitubers had reduced variation because microbial interactions were avoided by culture under axenic conditions, the effect of nutrients was consistent, and seasonal variations and other environmental variations were avoided. In most cases a single genotype was studied because subcultured plantlets were all descended from one parent plant (although this is also the case in field grown cultivars because they are vegetatively propagated as clones). Other advantages of using minitubers were that plantlets were quicker to grow and produce tubers than field grown plants (three months instead of at least six months) and plantlets could be grown continuously all year round. Disadvantages included the possibility of altered regulation caused by an unnatural habitat. The minitubers were grown at about one third the intensity of full sunlight, however even at this light intensity the amount of anthocyanin produced appeared to be reaching a plateau (Figure 5.14), and it is unlikely that higher light intensities would have significantly increased the concentration of anthocyanins produced.

For these minitubers, the concentrations of phenolic acids, flavonoids, and particularly anthocyanins were best presented as ng/cm^{2}SA rather than μg/gFW. This was because the minitubers were too small to be peeled and because the skin and flesh could not be separated, whole minitubers were analysed. This was especially useful for anthocyanins because, in the minitubers of all cultivars studied, anthocyanin was present only in the skin, with none found in the flesh. Flavonoids and phenolic acids were found in the flesh as well as the skin, although because such low concentrations were found in the flesh, these were probably insignificant in the small minitubers used because of the large SA to volume ratio.

5.5.2 Regulation: transport of "trigger" or carbohydrate?

The original nodal position of the explant affected not only the size of minitubers produced, but also the amount of anthocyanin synthesised in the minituber. Plants from higher nodes (closer to the apical meristem) produced fewer, larger minitubers with higher concentrations of anthocyanin (Table 5.4). It was thought that this greater amount of anthocyanin in minitubers from higher nodes maybe directly related to the size of the minitubers. This is because it was found previously (Chapter 4) that there was a good correlation between tuber size and anthocyanin concentration. The higher anthocyanin concentration and larger minitubers from the top node (N1) may have been because of the growth aspects of the explant. The top nodal cutting already contained an apical meristem and a number of unexpanded leaf pairs, and thus, the structure of the growing explant was already formed and only had to expand. The lower nodes of the original plantlet (N2 and N3) consisted of only one leaf pair, and grew a new plantlet from the axillary bud. Differences in anthocyanin concentrations may be related to carbohydrate metabolism and competition for carbohydrates. This is because the top nodal cutting had a greater FW and was more likely to contain higher reserves of carbohydrate, surplus from primary growth, and therefore available for secondary metabolism. To confirm this hypothesis it would have been useful to measure the levels of carbohydrate in each of the nodal cuttings. However, because this work was carried out towards the end of this thesis, time did not permit this investigation.

After two weeks growth, nodes towards the top of the plantlet produced a higher total tuber weight per plant, suggesting that more carbohydrate was available for tuber growth (and anthocyanin biosynthesis), rather than having to be used in the growth of the new stem and leaves. Additionally, Creasy (1968) found that photosynthesis and carbohydrate metabolism were extremely important in controlling the synthesis of anthocyanins in strawberry leaf disks. However, in the work carried out in this thesis, an exogenous source of carbohydrate (sucrose) was supplied in the medium, so it is not likely that carbohydrates were limiting. Research into the carbohydrate metabolism of tissue-cultured plantlets has shown that some plants have very low levels of photosynthesis, and that active growth is dependent on the exogenously supplied carbon source (reviewed in Conner and Thomas, 1981; Donnelly *et al.*, 1984). Therefore, this points to the involvement of some other "trigger" compound being produced by the apical tissues or leaves, to regulate anthocyanin biosynthesis, rather than carbohydrate from photosynthesis. This aspect obviously needs further investigation, but was outside the scope of this study.

Most of the experiments using minitubers were carried out with the cultivar Desirée. It was found that a two week dark incubation period immediately after the plantlets were transferred to TIM (tuber initiation media), before returning the plantlets to the light, increased the number of minitubers. However, a large decrease (43%) in individual tuber weight was observed, with the total tuber weight decrease of 33% per plant (Table 5.5). For this cultivar it was found that plantlets grew successfully for two weeks in the dark, and after transfer to TIM were capable of further growth in the dark for nine weeks and production of minitubers (because of the availability of the external carbohydrate source). In general, the amount of light present while the plantlets were growing in PMM (potato multiplication media) determined the number of minitubers formed, whilst the intensity of light present while the minitubers were developing in TIM determined the average weight of each minituber. Additionally, light conditions during the growth the plantlet in PMM had no effect on the concentration of anthocyanins, flavonoids or phenolic acids; only the light conditions while the minituber was growing (in TIM) were important.

Desirée minitubers grown in the dark did not contain any anthocyanins. At first this was surprising because field grown Desirée tubers (under ground in the dark) produced large quantities of anthocyanin. Reasons for the complete lack of anthocyanin biosynthesis in the dark grown Desirée minitubers appeared to be that the plants do require light for anthocyanin production in the tubers. That is, although the tubers do not require direct light, it is proposed that the photosynthetic parts of the plant must be exposed to light for anthocyanin biosynthesis to occur in tubers. This could be for two reasons 1) the exposure of the aerial parts of the plant to light induces the production of some "trigger" molecule (perhaps a hormone or co-factor) which is transported down to the tubers to initiate anthocyanin biosynthesis and/or, 2) anthocyanin biosynthesis may be associated closely with carbohydrate metabolism, in that plants in the dark do not photosynthesise. Therefore, there is a lack of carbohydrates for plantlet and minituber growth and carbohydrate is diverted towards the primary pathways at the expense of secondary metabolism, such as anthocyanin biosynthesis.

Presumably, other parts of the flavonoid pathway (other than the anthocyanin branch) were active in dark grown minitubers, because low concentrations of flavonoids were found in minitubers grown in the dark. However, flow through these flavonoid pathways was also increased in the light, and light grown minitubers contained three times the concentrations of flavonoids, compared with the dark grown minitubers (Table 5.6). Dark grown minitubers also contained moderate concentrations of phenolic acids and, although these also increased in the light, the increase was much smaller (increased by 30% in the light). Therefore, although the exposure of the minitubers to light increased

the biosynthesis of all these phenolic classes (phenolic acids, flavonoids and anthocyanins) the magnitude was vastly different. This suggests either 1) that the precursor is used up in the earlier parts of the pathway (*i.e.* phenolic acid production) and/or, 2) that different control mechanisms exist for the biosynthesis of phenolic acids, flavonoids and anthocyanins. The latter is supported by the fact that the anthocyanin pathway was completely inactive in the dark (in Desirée minitubers), whereas the other two pathways (to produce phenolic acids and the other flavonoids) showed at least some activity (judged by the appearance of end products). Thus, the anthocyanin pathway was inactive, but the flavanones (eriodictyol and naringenin) and the flavan-3-ol (catechin) were found in dark grown minitubers. Therefore, the phenylpropanoid pathway producing the flavonoid precursors was active. Additionally, the branch of the flavonoid pathway producing catechin was also active, which suggests that naringenin and eriodictyol intermediates were converted into catechin instead of anthocyanins (Figure 5.4).

The total lack of anthocyanin production in dark grown Desirée minitubers was surprising. Therefore, similar experiments were carried out with tissue-cultured plantlets from other cultivars (Amadeus, Bildtstar, RKE and Urenika). The two light pink cultivars (Amadeus and Bildtstar), which contained a similar concentration of anthocyanins to Desirée in the light, also showed very little, or no anthocyanin production by minitubers in the dark. However, cultivars with higher concentrations of anthocyanin in the light (RKE, three times, and Urenika, nine times the concentration of anthocyanin found in Desirée tubers) showed appreciable quantities of anthocyanin in the dark, although production in the light was still three to seven times higher than the production in the dark (Table 5.7). These results showed that anthocyanin production was possible in minitubers in the complete absence of light (over the whole plant). However, to rule out stored metabolites or "trigger" compounds left in the initial explant, an attempt was made to subculture the plantlet twice in the dark (in PMM) before transfer to TIM, but this proved to be unsuccessful because these plantlets did not produce minitubers. These results show the problems associated with this study because three processes, tuber initiation and growth, carbohydrate metabolism and secondary metabolism, all interact and it is difficult to separate effects of these individual processes.

The amount of anthocyanin produced in the dark was related to the anthocyanin producing capacity of the cultivar. It is possible that very small quantities of anthocyanin existed in Desirée minitubers grown in the dark, but were not detected. However, both diode-array spectrophotometric and HPLC methods were used in the attempt to detect any anthocyanin, and samples were concentrated as much as practicable, so this is not likely.

By covering the minitubers with foil, the effect of exposing stems and leaves to light, on anthocyanin, flavonoid and phenolic acid biosynthesis in tubers developing in the dark could be determined, and the effect of transported carbohydrates or a "trigger" compound investigated further. The anthocyanin concentration of minitubers in the light had increased to a maximum after two weeks, whereas in the covered minitubers the anthocyanin concentration increased from 0 to 40% of this maximum in two weeks, and increased further to 90% of the maximum after three weeks. Therefore, exposure of the photosynthetic tissues caused increased synthesis of anthocyanin in the minitubers, even though the actual minituber tissue was not exposed to light. These results also suggested that some compound is transported from the leaves to the tubers enabling anthocyanin production.

In these experiments with foil covered minitubers, the biosynthesis of the flavonoids (other than anthocyanins) showed a similar pattern to the anthocyanin biosynthesis. However, this was to a lesser extent, with the flavonoid concentration of foil covered minitubers increasing from 20 to 60% of that of minitubers grown in the light for three weeks. This suggests that the control of the flavonoid metabolism following exposure to light preferentially involves the use of flavonoid intermediates (naringenin-chalcone and naringenin) in anthocyanin biosynthesis, rather than along the other flavonoid pathways to produce flavones or flavonols.

The phenolic acid concentration increased two to three times in the light exposed minitubers, but there was no significant change in the phenolic acid concentration in the foil covered minitubers. This suggests that either the enzymes, or the synthesis of the enzymes, involved in the conversion of cinnamic acid and *p*-coumaric acid to other phenolic acids are not increased by exposure of the leaves to light. Alternatively, these enzyme activities are increased, but all phenolic intermediates are converted into flavonoids and anthocyanins, which appears to be most likely. Further investigation into whether these intermediates were used preferentially in anthocyanin biosynthesis would have been useful, but there was not time to carry out this work during the time frame of this PhD.

The formation of anthocyanins and flavonoids in foil covered minitubers took place over a longer time than in those minitubers which were directly exposed to light, perhaps indicating the longer time for transport of effector molecules. It is interesting that the anthocyanin concentration in covered minitubers reached almost the same levels as in light exposed tubers (and may have done if they were left for a longer time). This suggests that this cultivar (Desirée) has a certain threshold, or maximum limit, of anthocyanin production for a given light intensity, independent of whether the whole

plant, including the minituber, or only the leaves, are exposed to light. Further work on this system, including the measurement of phenolics over a longer time frame to determine their equilibrium concentrations, and measurement of enzyme levels and activities, mRNA levels, and residual carbohydrate levels, would be useful in answering some of these questions. It would also be useful to alter the levels of carbohydrate present in the medium, and to measure the effects of including different hormones at various concentrations (after the minitubers were formed so as not to affect tuberization) on the anthocyanin concentration in minitubers, in the dark and light.

The distribution of anthocyanin in the minitubers showed the same pattern as that of small, developing field grown tubers (Chapter 4), with the anthocyanin being produced initially at the end closest to the stem. It was interesting to note that minitubers removed from the plantlet had less anthocyanin than those still attached under similar light conditions, and that the production of anthocyanin was not noticeably different between the stem and bud ends of these unattached minitubers (Section 5.4.8). These observations also indicate that the transport of some molecule(s) from the aerial parts of the plant is an important determinant of anthocyanin biosynthesis. It is also clear that the production of anthocyanin in the minitubers is influenced by the effect of light on the aerial parts of the plant more strongly than the effect of light on the tubers themselves. This is because minitubers attached to the plant, but covered with foil (*i.e.* in the dark), showed a greater increase in anthocyanin concentration than tubers themselves in the light, but not attached to the plant.

To investigate further the hypothesis of the transport of some molecule from the aerial parts of the plant to the tuber as a "trigger" for anthocyanin biosynthesis, potato grafts between cultivars with red and white tubers were used. Scions from cultivars with red tubers were grafted onto rootstocks from cultivars with white tubers to see if the transport of any molecule from the "red top" would induce anthocyanin biosynthesis in the "white tubers". However, this did not occur and the "white tubers" remained white. Furthermore, scions from a cultivar producing white tubers were grafted onto rootstock from a cultivar with red tubers, and the anthocyanin production remained the same. Inferences that can be made from these results are 1) that if a compound is transported from the shoots which is necessary to initiate anthocyanin biosynthesis, it is produced in both white and red genotypes and, 2) the tuber must be genetically competent to produce the anthocyanin biosynthetic enzymes, and it is not the lack of substrate or some "trigger" compound which is preventing the production of anthocyanins in white tubers. That is, the initiation of anthocyanin production is controlled in the tuber, not in the shoot, even though an effector molecule from the shoot may be required.

5.5.3 Effect of light

It has been found that exposing the dark grown minitubers to light increased the production of anthocyanins: but how much light is needed, for what length of time and what light receptors and responses are involved? To answer some of these questions Desirée minitubers were grown in the dark (LG/DT), exposed to various lengths of time in the light, then incubated in the dark for the remainder of a two week period to allow time for anthocyanin production. A significant increase in anthocyanin concentration occurred after eight hours of light incubation (Figure 5.17). Shorter times did not result in significant increases in anthocyanin synthesised by the end of the two week period, and longer times resulted in a linear increase of anthocyanin with time. This suggests that anthocyanin biosynthesis in potato tubers is photo-regulated by a high irradiance response (HIR). There was no detectable response to short bursts of light, which suggests that this form of photo-regulation was not responsible (or not detected).

HIR's are typically caused by exposure to red/far-red and/or UV-A/blue light (Stafford, 1990). To determine the type of photoreceptor acting in this HIR, the effect of light at different wavelengths was investigated. However, the changes in anthocyanin concentrations in these experiments were partially caused by differing light intensities, rather than just the different wavelengths. Nevertheless, exposure to both blue and red light, and to a lesser extent purple light, increased the anthocyanin concentration compared with white light at the same intensities (Figure 5.16). Also, exposure to UV and far-red light increased the anthocyanin concentration, over that of the dark control (Figure 5.15). However, the spectra of light passing through these filters was not measured, and the increase may have been caused by an increase in photosynthesis rather than the photo-induction of anthocyanin biosynthesis. This was not thought to be the case, however, because tuber numbers and weights corresponded to those of tubers grown in the dark, whereas the anthocyanin concentration was increased, and as mentioned previously tissue-cultured plantlets tend to have low levels of photosynthesis even in full light (Conner and Thomas, 1981; Donnelly *et al.*, 1984). Thus, these results suggest that both UV/blue and red/far-red photoreceptors (cryptochrome and phytochrome) are capable of acting to bring about the increase in anthocyanin in minitubers, as is thought to be the case in most HIR's (Stafford, 1990).

5.5.4 Enzyme activities and phenolic biosynthesis in minitubers

The effect of light on enzyme activities and comparison with concentrations of anthocyanins, flavonoids and phenolic acids was investigated to determine the influence of light regulation on the biosynthesis of these compounds. After exposure of dark

grown minitubers to light for varying lengths of time, the anthocyanin concentration showed a linear increase up to a maximum anthocyanin concentration of 3ng/cm^{2}SA in ten days, after which there was no change (Figure 5.18a). A similar maximum anthocyanin concentration in Desirée minitubers was found in a number of different experiments. Flavonoid and phenolic acid concentrations increased rapidly up to a maximum in about four to five days, after which the rate of production of flavonoids and phenolic acids was reduced with no further significant increases (Figure 5.18b and c).

The enzyme activities of Desirée minitubers before exposure to light were lower than those of field grown Desirée tubers (Section 3.5.2) however, after light exposure, enzymes activities of minitubers were increased. Dark grown minitubers contained reasonably high background levels of PAL activity, although after exposure to light, the activity levels increased dramatically by 2.5 times in the first 20h, after which levels slowly decreased (Figure 5.19). The increase in PAL activity after light exposure is well known (Lamb, 1979; Wilkinson and Butt, 1992; Reddy *et al.*, 1994), and because PAL is the first enzyme in the phenylpropanoid pathway, its early rapid increase is not surprising. The level of PAL activity at this early stage correlated well with the rate of anthocyanin production, which was shown to have significantly increased after eight hours of light exposure (Figure 5.17).

The next enzyme in the phenylpropanoid pathway, C4H, had no detectable activity for the first two days (Figure 5.20), which was surprising because flavonoid and anthocyanin concentrations increased during this time (Figure 5.18). It is likely that low levels of C4H activity were present during this early stage, but were not detected using the method employed. C4H activity subsequently increased rapidly to a maximum at about nine days and then decreased. This was closely related to the rate of increase of anthocyanins, flavonoids and phenolic acids.

The second enzyme in the flavonoid pathway, CHI, also had no detectable activity before exposure to light, but appreciable activity was measured after light exposure. Activity increased to a maximum at about eleven days, and then decreased to zero after twenty five days (Figure 5.19). This corresponded almost exactly with the rate of anthocyanin increase, with anthocyanin produced at a relatively constant rate for eight days, followed by a decreased rate of production up to about ten days, after which there was no further increase in anthocyanin concentration.

Activities of all the other enzymes involved in flavonoid and anthocyanin biosynthesis (F3H, F3'H, DHR and GT) also showed increased activity after light exposure. It is interesting to note that the two early enzymes in the phenylpropanoid pathway (PAL and

C4H) reached maximum activities first (20h and 9 days respectively), whereas the later enzymes of the flavonoid pathway reached maximum activities after longer exposures to light.

High GT activities were found with the substrates Km and Qu, which were commonly found in tubers (Appendix 3), however with luteolin, which was not found in tubers in this study, activities were low. Out of the three sugars tested (UDP-glucose, UDP-galactose and UDP-xylose), UDP-glucose had the highest activity, as was expected because all the flavonoids identified in tubers had at least one glucose unit attached (Chapter 3). Surprisingly however, there was a significant amount of activity with UDP-galactose as a substrate, when there is no evidence of any flavonoids in potatoes containing galactose, either in this work, or the work of other researchers (Harborne, 1960a; 1967). Therefore, the sugars found attached to the flavonoids in potatoes are not determined by the specificity of the GT's, but are perhaps controlled by the availability of the sugar substrates. The other sugar (besides glucose) which is commonly found in the flavonoids of potatoes is rhamnose, however it was not possible to test the activity of GT with this sugar because of the lack of commercial availability of UDP-rhamnose.

5.5.5 Conclusion

In summary, anthocyanins, flavonoids and phenolic acids all showed increased production after exposure to light. Anthocyanins showed a significant increase after eight hours and reached a maximum of about 3.2ng/cm^{2}SA after about ten days. Flavonoids and phenolic acids reached maxima after about four to five days. Enzyme activities of PAL, C4H, CHI, F3H, F3'H, DHR, and GT were closely related to the rates of synthesis of these phenolics. No anthocyanin was produced in Desirée minitubers grown in the dark, although anthocyanin was produced in dark grown minitubers of cultivars with a higher anthocyanin concentration (RKE and Urenika). However, for full production of anthocyanin, the aerial parts of the plants (not necessarily the tubers) required light. It appears that some compound, probably a "trigger" compound (e.g. a hormone, co-factor or metabolic intermediate) was produced or activated in the leaves as a result of light exposure, and was transported to the tubers. It was proposed that, in order to produce anthocyanins the tubers 1) need to be genetically competent, 2) require an adequate carbohydrate source as well as, 3) require the presence of a "trigger" compound, however some cultivars (e.g. Urenika) produce limited amounts of tuber anthocyanin in the absence of light (*i.e.* "trigger" compound).

CHAPTER 6

Morphological studies - distribution of anthocyanin containing cells

6.1 Introduction

6.1.1 Structure of mature tubers

The mature tuber is composed of a number of layers:- periderm, primary cortex, vascular ring, and pith, each of which is composed of a number of different cell types. There appears to be some confusion in the literature in the naming and presence of various layers, so the following nomenclature will be used. The outer most layer of a mature potato tuber consists of the periderm layer, commonly called the skin (Cutter, 1978; Peterson *et al.*, 1985). The periderm is usually 6-10 layers thick, and is a specialised protective layer to prevent water loss and impede attack by various soil pathogens. The periderm is composed of phellem (cork), phellogen (cork cambium), and phelloderm (secondary cortex) tissues (Reeve *et al.*, 1969b; Lulai and Orr, 1994). Phellem tissues are the tangentially layered corky suberized cells on the tuber surface. Phellem cell walls are complex and consist of a middle lamella, a primary cell wall, a secondary cell wall with suberin lamellae which protects the tuber from water loss and disease, and a cellulosic tertiary cell wall (Schmidt and Schönherr, 1982; Vogt *et al.*, 1983). These cells may be senescent, with the outermost layers containing dead cells. Phellogen tissues are the next narrow layer of cells below the phellem, and these cambial cells actively divide outwards to form the phellem cells and inwards to form the phelloderm cells. The phelloderm is a narrow layer of secondary cortical parenchyma cells which forms below the phellogen. Inside the periderm is the primary cortex, which is also a narrow layer of cortical cells. The primary and secondary cortex separate the phellogen from the vascular tissue, and throughout this work both will be referred to as the cortex (or cortical layer), with no differentiation between the primary and secondary cortex. These cortical cells form a minor component of the mature tuber, but contain more starch grains per cell than other parenchyma cells in the tuber (Reeve *et al.*, 1970). The tuber has internal and external phloem developed from procambial tissue, which constitutes a larger proportion of the vascular tissue than the xylem. During tuber enlargement, the internal phloem becomes separated into numerous strands isolated by storage parenchyma (Figure 6.1) (Peterson *et al.*, 1985). The rest of the tuber, towards the centre, is made up of thin walled parenchyma (pith) cells.

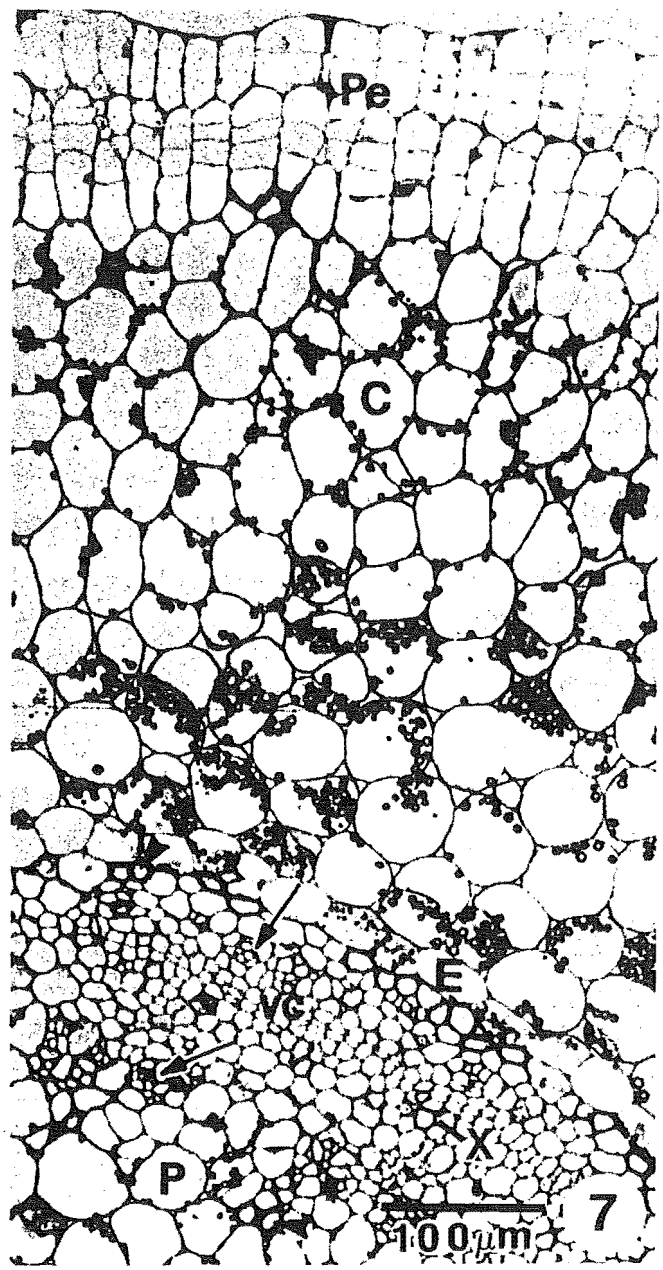


Figure 6.1 Primary tissue organisation in a minituber of cultivar Kennebec cultured *in vitro*. Periderm (Pe), primary cortex (C) with starch grains, endodermis (E), internal and external phloem (arrows), vascular cambium (VC), xylem (X) and pith (P). With the exception of the endodermis, the tissue organisation is identical to mature tubers grown *in vivo*. From Peterson and Barker (1979).

In potato tubers containing anthocyanin pigments only in the skin (and no colour in the flesh), the colour may be present in the phellem and/or cortex. In some cases (e.g. Arran Victory), the phellem is not coloured and the pigment is found in the cortex, whilst in other cultivars (e.g. King Edward VII), the phellem only is pigmented (Whitehead *et al.*, 1953; Burton, 1989). In the dead cells of the phellem, the anthocyanins must be residual, or impregnating cell walls because there are no living contents. Anthocyanin pigmentation in the flesh may occur only in the vascular tissue (e.g. Arran Victory), in the flesh inside the vascular ring (pith) (e.g. I53, Stage II Blue), or throughout the whole flesh (all cell types) (e.g. Urenika).

6.1.2 Objectives

The following microscopical studies were carried out to determine the location of anthocyanin containing cells, and to attempt to answer the following questions.

1) Do all the cells of a particular layer (*i.e.* in tangential sections) have the same intensity of colour?

2) What is the distribution of coloured cells? Are they evenly or randomly located, or are they clumped together in clusters in the tangential sections? This will of course depend on the pattern of colour of the tuber, whether the tuber is wholly or partially coloured, and if partially coloured the distribution of colour (refer to Section 1.2.2.1).

3) What determines the difference in colour intensity from weakly coloured to strongly coloured tubers? Is each individual cell more intensely coloured, or are there a greater proportion of cells with colour in the tangential section, or are the coloured cells present to a greater depth (*i.e.* more layers of coloured cells in the transverse section)?

4) Are all the cells the same colour, that is, are there any visible differences in the λ_{max}, perhaps caused by different chemical structures, or bathochromic shifts of the anthocyanins?

6.2 Methods

Tubers from a number of cultivars were selected to give 1) different tuber colours, either red or purple and, 2) different intensities of colour, so for each colour (red or purple), cultivars with a range from lightly to strongly coloured tubers were selected. For red tubers the cultivars Red Rocks, Desirée, Red Rascal and Red Flesh were used, showing a range from light to dark colour respectively, and for purple tubers the cultivars Tekau, Arran Victory, Blue Derwent, Stage II Blue and Urenika were used, with light to dark colours respectively.

Thin sections (consisting of at least three to four cells in thickness) were cut with a razor blade with the assistance of Dr Jan Grant (NZ Institute for Crop & Food Research Ltd, Lincoln). Sections of the tuber skin were either tangential (TG), parallel to the surface of the tuber, or transverse (TV), at right angles to the surface, with additional transverse sections cut from the flesh of cultivars with coloured flesh. Sections were viewed in areas which were of approximately four cells thick, because the size of cells differed between tissues and also between cultivars, and also so that intact cells could be viewed, not just the damaged cells on the cut surface of the section. Many sections were cut, and viewed under a light microscope with a 10x eyepiece and either a 4x or 10x objective magnification, to obtain a general impression of the characteristics and distribution of the coloured cells, and typical sections were photographed. All figures at the same nominal magnification are comparable because they were all photographed and processed in a similar manner.

6.3 Results

6.3.1 Pink/red tubers

The sections from Red Rocks, a light-pink skinned tuber (Figure 6.2), showed that no pigment was present in the cells or as residues in the dead outer layers of the phellem, or attached to the cell walls (Figure 6.3), and that only a very light coloured pigment could be observed in the lumen of the cells in the cortical layer (Figure 6.4). Desirée, a pink-red skinned tuber (Figure 6.5), had no colour in the dead cells of the phellem layer (not shown), but had more intensely coloured pink cells than Red Rocks in the cortical layer (Figure 6.6). In cells from the cortical layer of Desirée tubers, it was found that some cells were colourless, whereas others were more coloured; these coloured cells appeared to occur at random.

The presence of highly coloured "blobs" of pigment was observed in Figure 6.4 and Figure 6.6, and it was thought that these were not part of the cell contents but appeared to be dried residues which were thought to have come from the senescent cells of the phellem layer when it was removed from the cortical layer. It can be seen that the colour of these deposits varied from orange to red to purple, but it was not determined whether the difference in colour resulted from different pigment compositions, or from varying physiochemical conditions (e.g. pH). It is likely that a range of pH's would be found because of the different pH's of the cytoplasmic and vacuolar contents which would be released when the tissue was cut. Red Rascal tubers, with a similar coloured skin to

Desirée tubers, were also studied, and the cut sections (not shown) appeared to be almost identical to those from Desirée tubers.

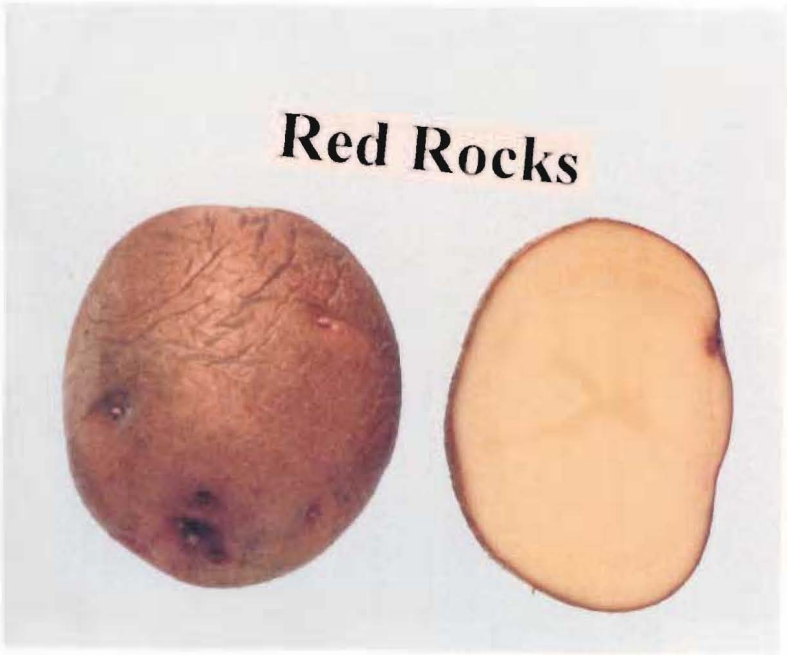


Figure 6.2 A typical Red Rocks tuber.

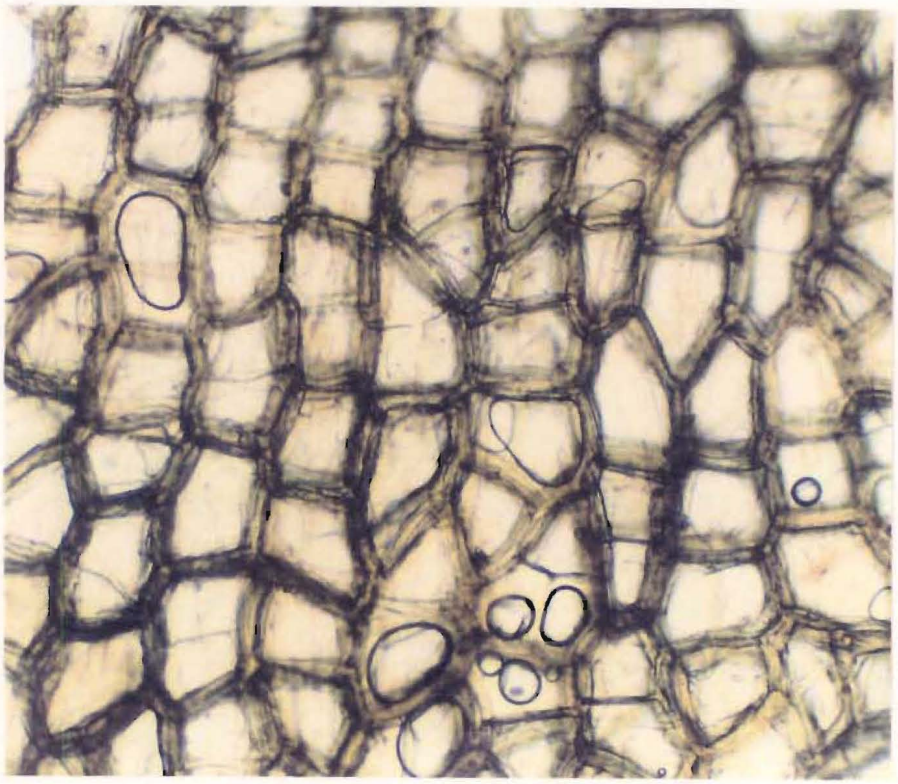


Figure 6.3 A typical tangential section of phellem from Red Rocks tubers.

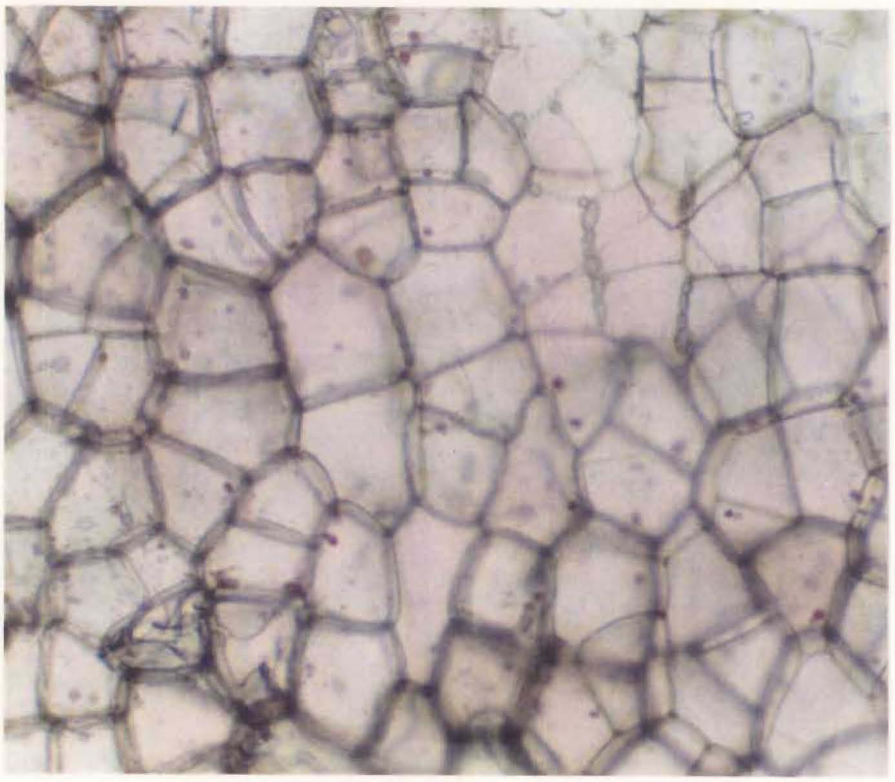


Figure 6.4 A typical tangential section of cortex from Red Rocks tubers.

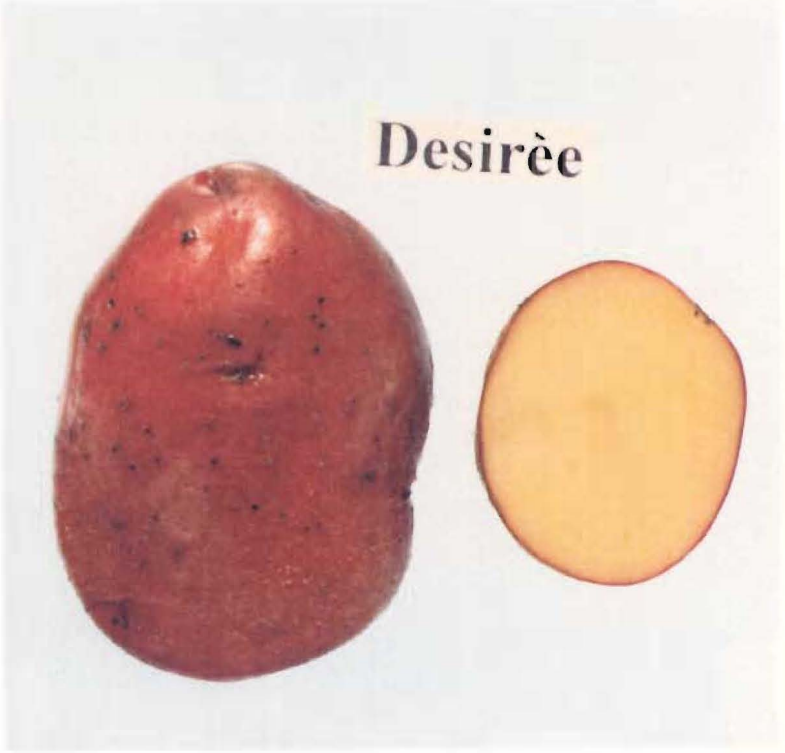
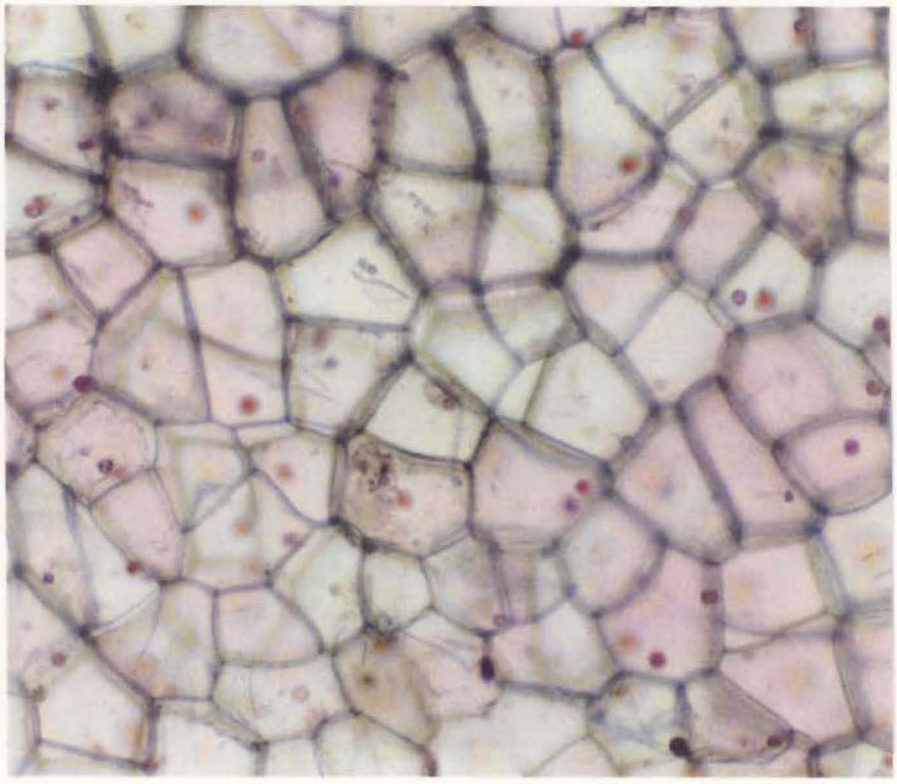


Figure 6.5 A typical Desirée tuber.



_____ = 50μm

Figure 6.6 A typical tangential section of cortex from Desirée tubers.

Red Flesh is a cultivar whose tubers have a dark red skin and pink flesh (Figure 6.7). Tangential sections of the phellem (Figure 6.8) showed what appeared to be intensely coloured deposits of anthocyanin around the cell walls of the dead phellem cells. It is thought that when the cells died, the vacuolar contents (including the anthocyanins) dried out and became deposited along the inside of the cell walls, thus contributing to the intense colour of the tuber. The pink colour of the cells in the cortical layer from Red Flesh tubers (Figure 6.9) was slightly darker than that found in similar sections of Desirée tubers (Figure 6.6). However, from observation of transverse sections of both Desirée and Red Flesh (not shown), it appeared that number of layers of coloured cortical cells (3-5 layers) was similar in both cultivars. There appeared to be a thick outer-most layer of highly coloured dead phellem cells in Red Flesh in contrast to Desirée or Red Rocks (not shown), where there was no colour in this layer. Therefore, the much darker red colouration observed in the skins of Red Flesh tubers, compared with that of Desirée tubers, was due mainly to the deposits of anthocyanin in the dead cells of the phellem layer.

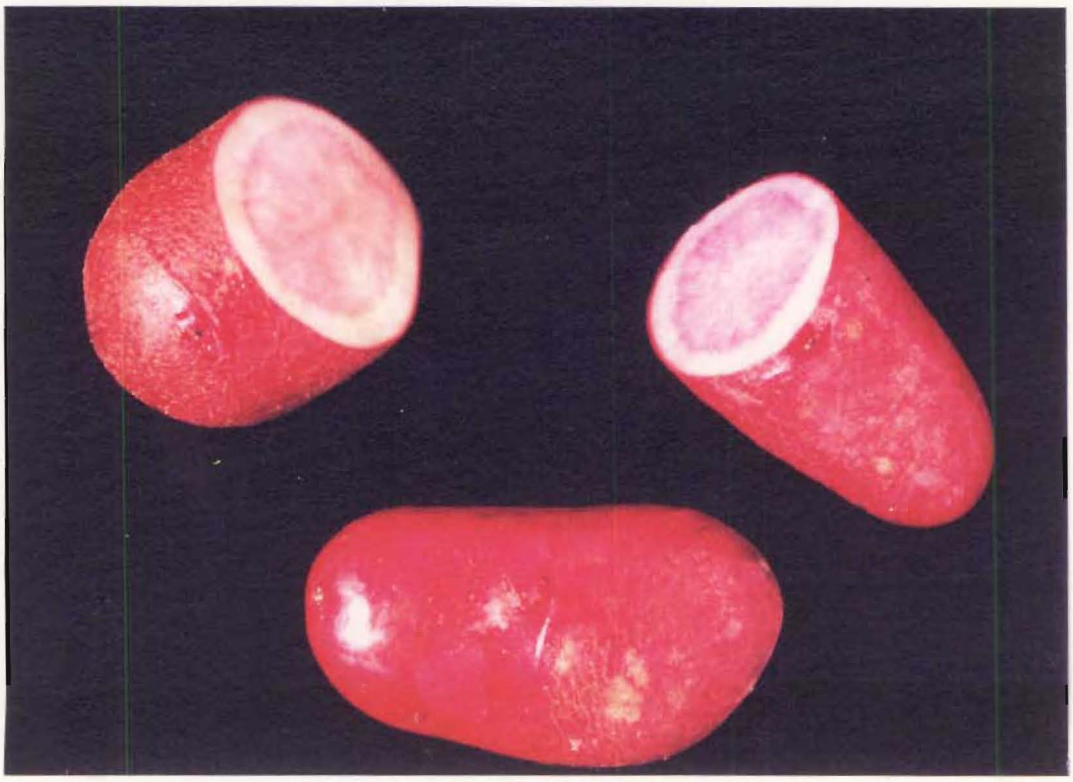


Figure 6.7 A typical Red Flesh tuber.

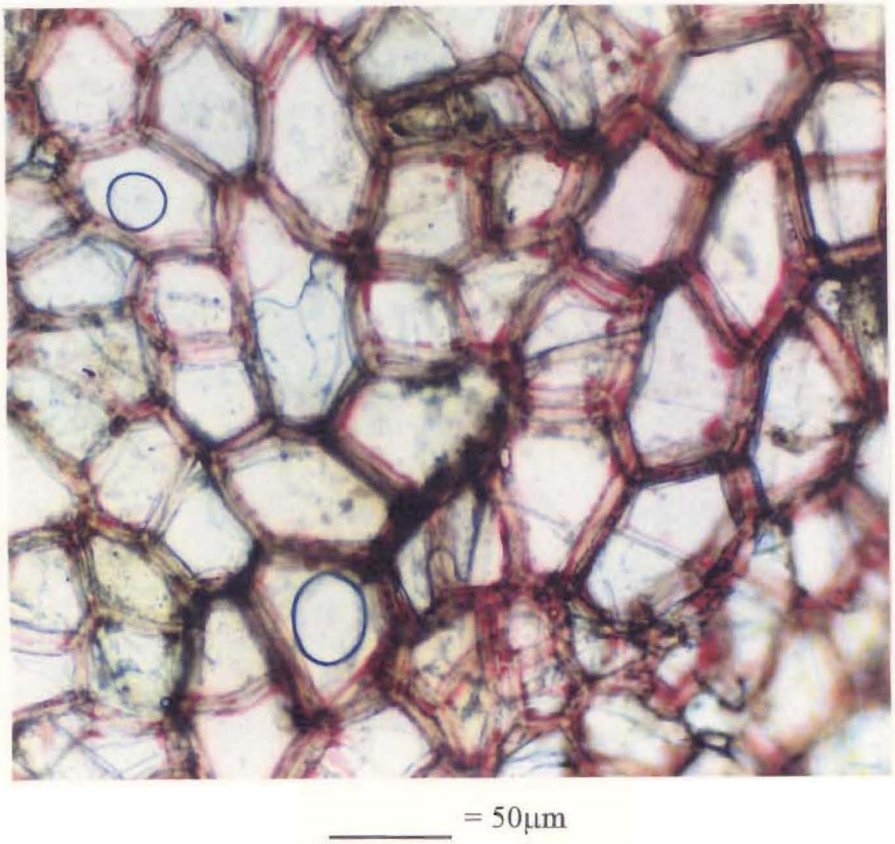
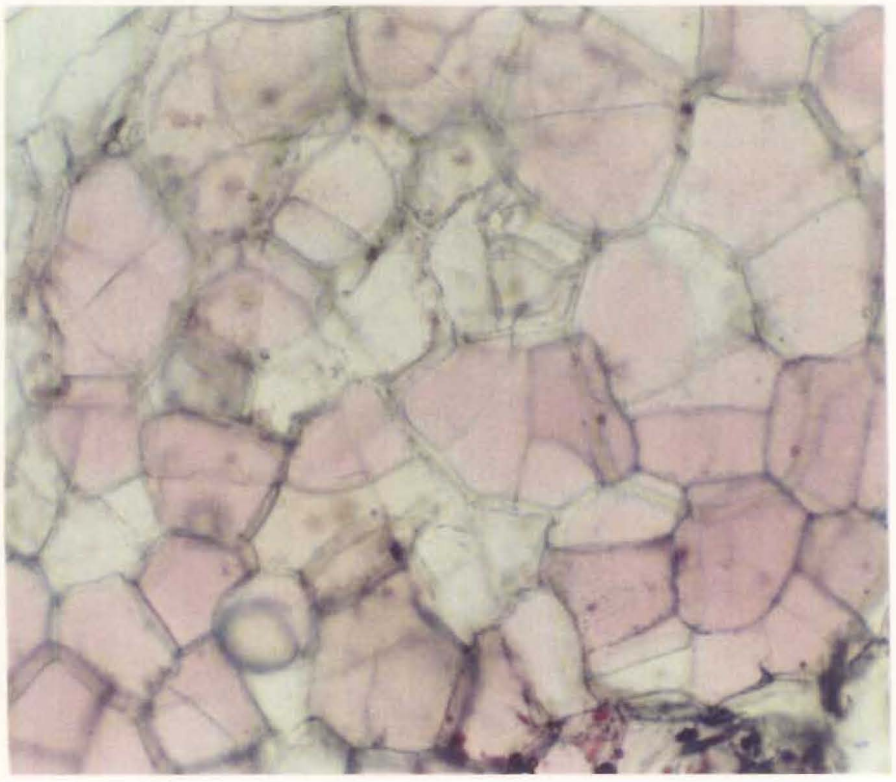


Figure 6.8 A typical tangential section of phellem from Red Flesh tubers.



_____ = 50μm

Figure 6.9 A typical tangential section of cortex from Red Flesh tubers.

6.3.2 Purple tubers

Tubers from the cultivar Tekau were mostly white, but showed small patches of purple pigment particularly around the eyes (Figure 6.10). Transverse sections, cut from tissue around the eyes, showed that the coloured cells were clustered together in the cortical layer, with little or no colour in the phellem cells (Figure 6.11). Tangential sections from Tekau tubers showed that the coloured cortical cells showed strong purple colouration, whilst the other cells were colourless, and that this was not an artefact of sectioning (Figure 6.12). It is clearly obvious that the coloured anthocyanin (typically found in the vacuole) takes up almost all of the cell lumen (Figure 6.12).

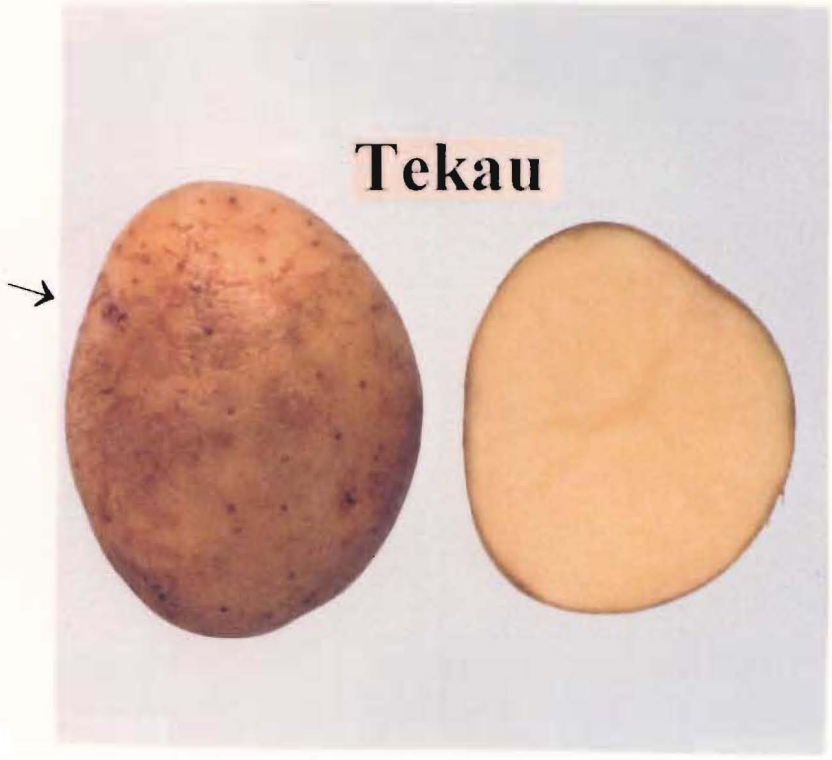


Figure 6.10 A typical Tekau tuber. Arrow points to an eye with purple colouration.

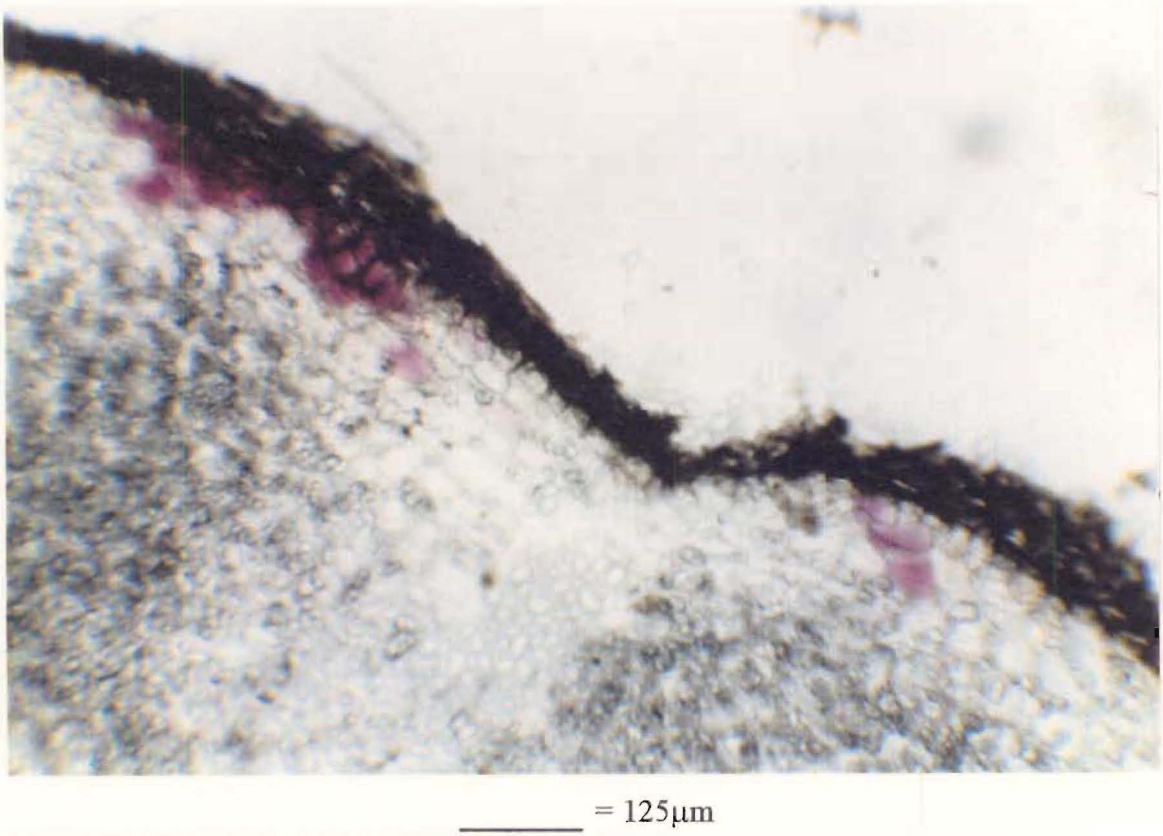


Figure 6.11 A typical transverse section from Tekau tubers, tissue cut through an eye.

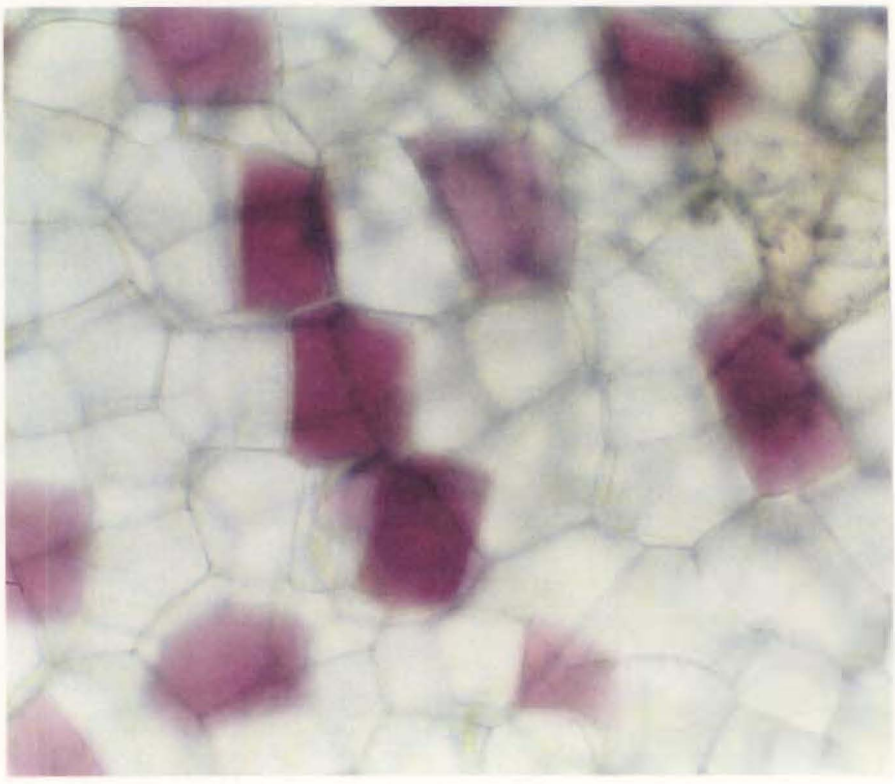


Figure 6.12 A typical tangential section of cortex from Tekau tubers.

Arran Victory tubers had a light purple skin and purple ring of vascular tissue (Figure 6.13). Tangential sections of the cortical layer of Arran Victory (not shown) were similar to that shown in Figure 6.9 from Red Flesh sections, except that the colour was more purple. The coloured cells appeared to be arranged in a random manner, except that around the eyes there was a higher density of coloured cells, and these coloured cells appeared to be more clustered. There was no anthocyanin in the dead cells of the phellem layer of Arran Victory tubers (not shown). Transverse sections of Arran Victory flesh, cut through the vascular tissue, showed the anthocyanin present in the cells in the vascular area (Figure 6.14), and it was observed that the colour varied from blue to purple. The presence of starch grains in these tissues slightly obscured the details of the cells in this photograph (Figure 6.14), however the patches of colour appeared to be within intact cells. It is possible that the differences in colour may be caused by different pigments, or that different cells may naturally contain pigment at different colours because of changes in pH or copigmentation. Phloem sap is usually more alkaline (pH 7.5-8.6) (Street and Öpik, 1970) in contrast to other plant cells (pH 2.5-7.5) (Stewart *et al.*, 1975), and this may have produced the bluer colours in vascular tissues as observed in Figure 6.14.

Figure 6.13 A typical Arran Victory tuber.

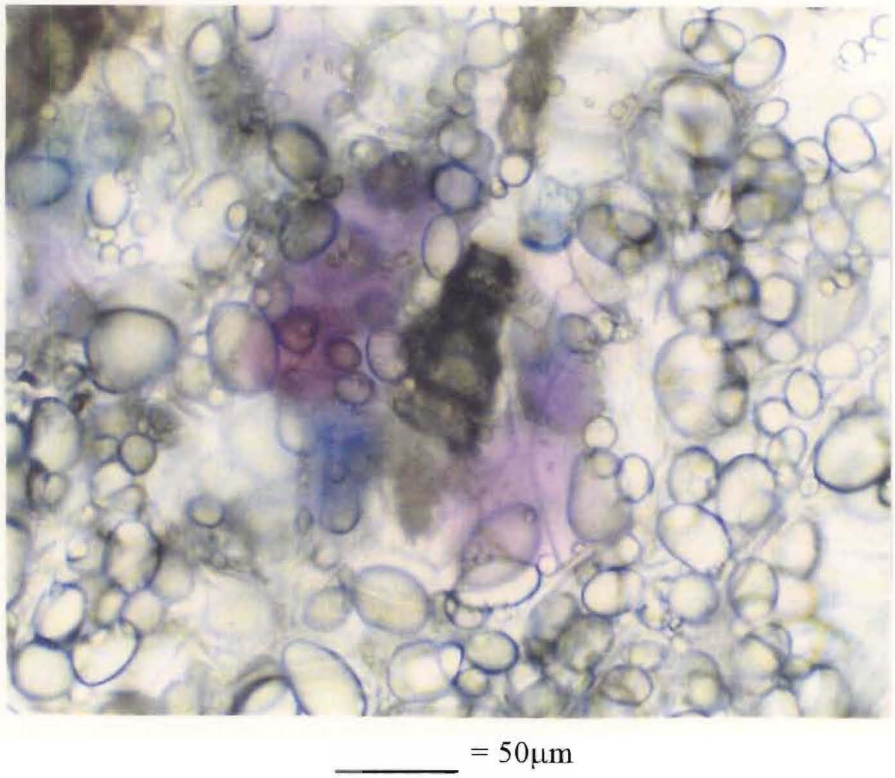


Figure 6.14 A typical tangential section of tuber flesh from Arran Victory tubers cut across the vascular tissue.

Blue Derwent tubers (Figure 6.15) had a slightly darker purple skin than Arran Victory tubers. Transverse sections of Blue Derwent skin (periderm), showed that the coloured cortical cells extended to a depth of three to four cells deep (Figure 6.16), as did those of Arran Victory (not shown). Tangential sections of the cortical layer from Blue Derwent (Figure 6.17) were similar to those of Arran Victory tubers (not shown). The colour of the cells ranged from colourless to a light purple, and again the coloured cells appeared to be distributed in a random manner, except around the eyes where there were a larger number of coloured cells.

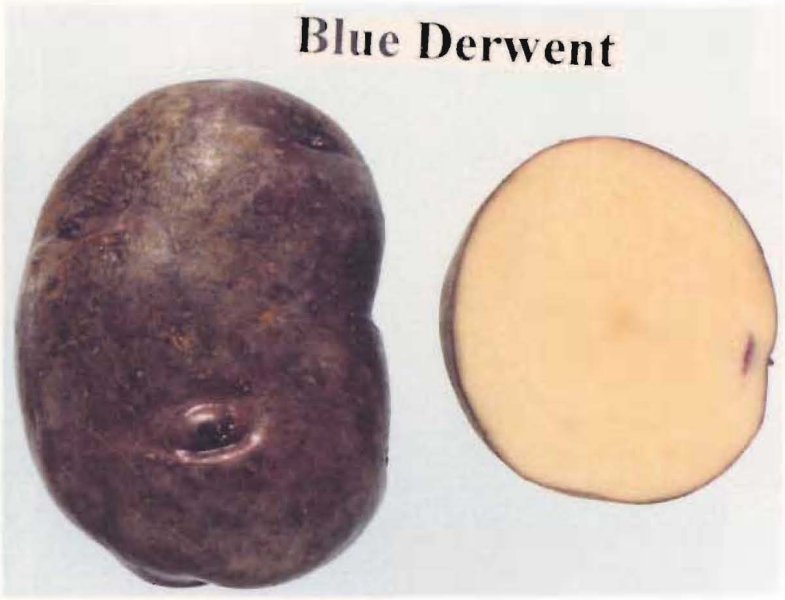


Figure 6.15 A typical Blue Derwent tuber.

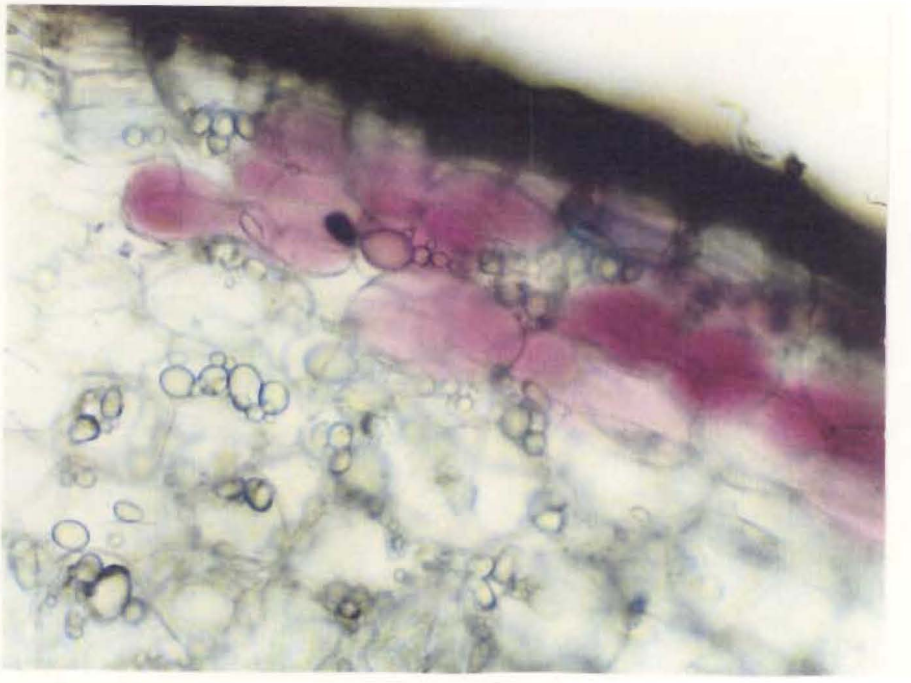
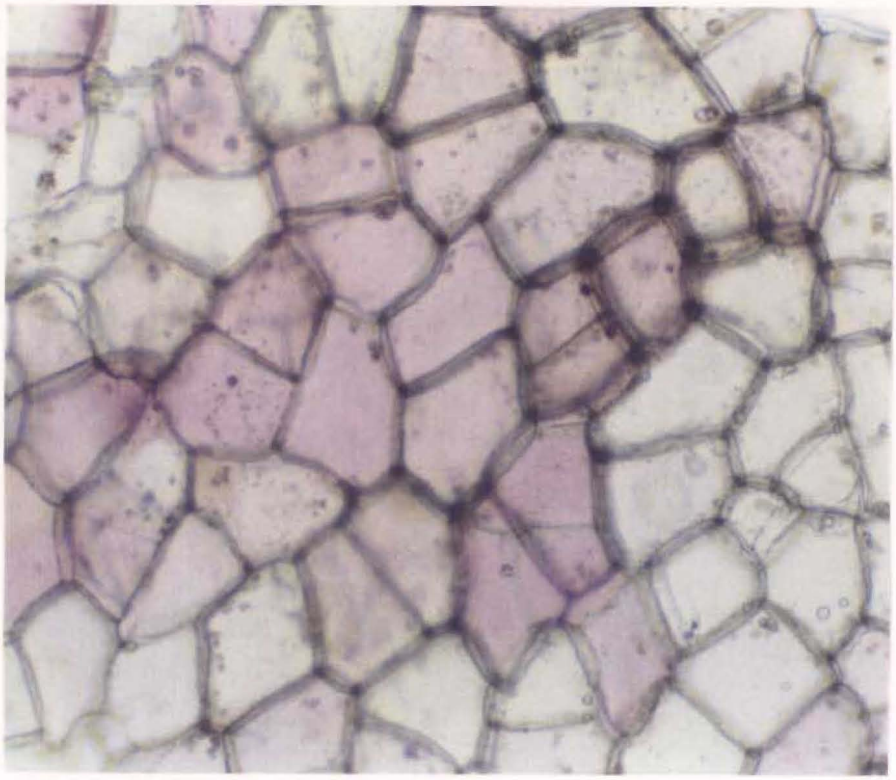


Figure 6.16 A typical transverse section from the surface from Blue Derwent tubers.



_____ = 50μm

Figure 6.17 A typical tangential section of cortex from Blue Derwent tubers.

Stage II Blue tubers had a dark purple/black skin, with some purple colouration in the flesh, in the region of and inside the vascular ring (Figure 6.18). The colour of cells in the cortical layer from Stage II Blue tubers (Figure 6.19) was much more intense than that found in the lighter coloured skins of Arran Victory tubers (not shown) and Blue Derwent tubers (Figure 6.16). However, there was a complete range of colour intensities in the cells of the cortical layer of Stage II Blue tubers, with the colour of cells ranging from colourless through light and medium shades of purple, to a fairly dark purple colour. Transverse sections of Stage II Blue tuber skins (Figure 6.20) also showed increased colour intensity when compared with similar sections from the lighter coloured Arran Victory tubers (not shown) and Blue Derwent tubers (Figure 6.17). Additionally, there was an increased number of layers of cortical cells containing anthocyanin pigmentation, with the coloured cells present to a depth of five to seven cells in transverse sections in Stage II Blue (Figure 6.20), compared with three to five cells in Arran Victory (not shown) and Blue Derwent (Figure 6.17).

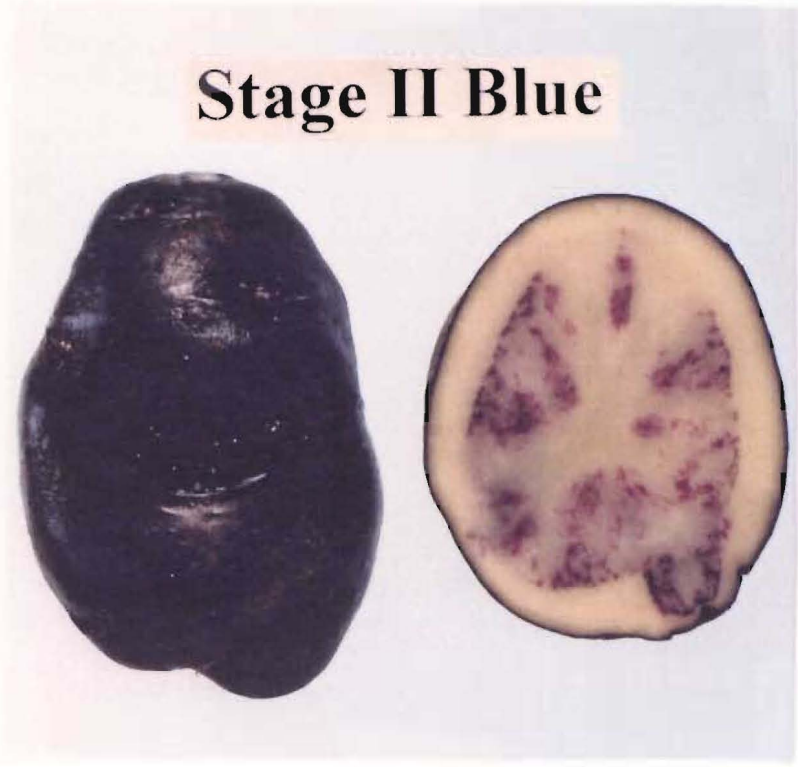


Figure 6.18 A typical Stage II Blue tuber.

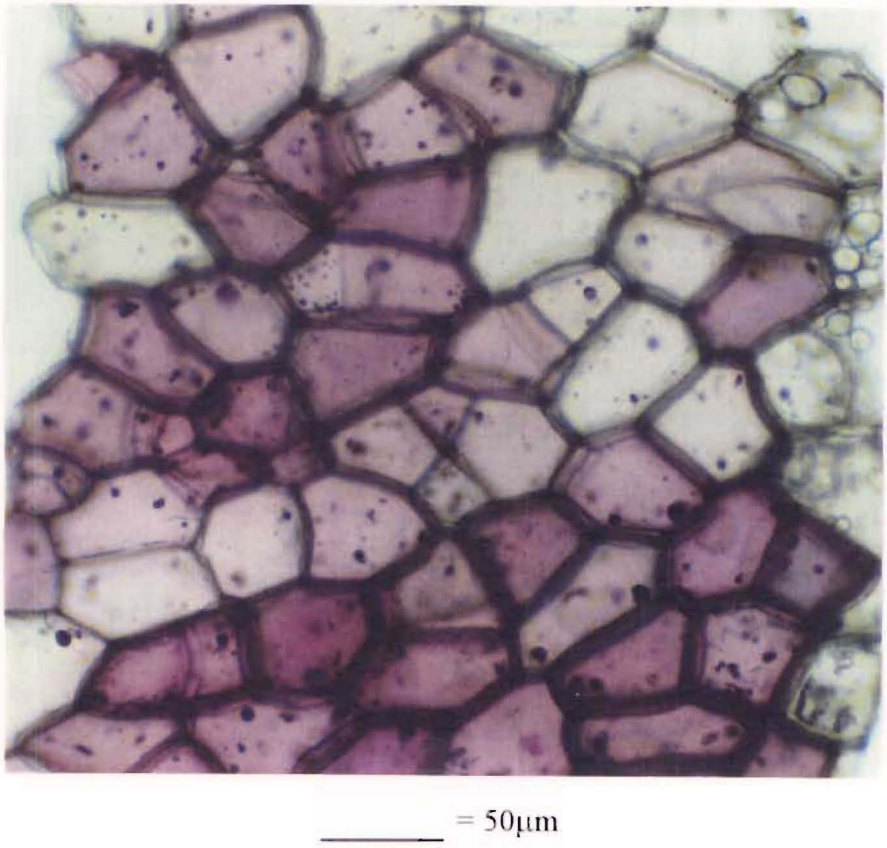


Figure 6.19 A typical tangential section of cortex from Stage II Blue tubers.

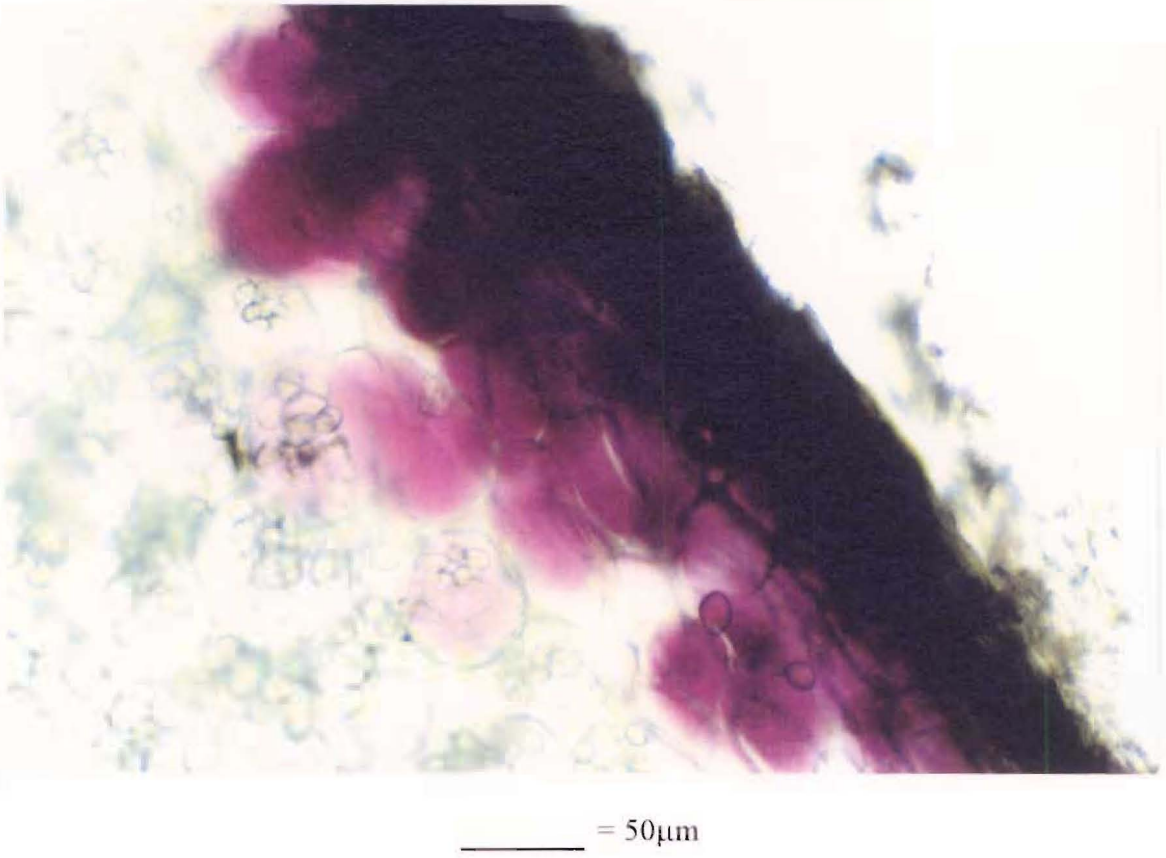


Figure 6.20 A typical transverse section from Stage II Blue tubers.

Urenika tubers (Figure 6.21) had a similar skin colour to those of Stage II Blue, but were typically a dark purple colour throughout the flesh. Tangential sections of the cortical layer of Urenika tubers (not shown) were similar to those found from Stage II Blue sections (Figure 6.19). Tangential sections Urenika of live phellem cells (Figure 6.22) were similar to those of Stage II Blue (not shown), and contained purple colour in the lumen of the cell (*i.e.* the vacuole), as well as dark purple deposits around the cell walls. Dead phellem cells (*i.e.* cells with no contents) of both these cultivars contained intense purple colour deposited around the cell walls (not shown). Transverse sections from Urenika of the periderm and underlying cortical cells showed that the pigmented cells continued from the phellem layer through to the pith cells (Figure 6.23). In transverse sections of Urenika pith (flesh) the cellular structure and presence of pigment was not clear because of the numerous starch grains (Figure 6.24), however the intensity of colour was not as great as might have been expected from the colour of the flesh when the tuber was viewed without magnification (Figure 6.21). It is thought that much of the anthocyanin had leached out of these cells, because of the greater structural damage of the thin walled pith cells when sections were cut, particularly with the high density of starch grains which were pushed aside as the blade passed through the tissue and which

may have damaged the cells. The lower undamaged layers could not be observed because of the large number of starch grains.

Figure 6.21 A typical Urenika tuber.

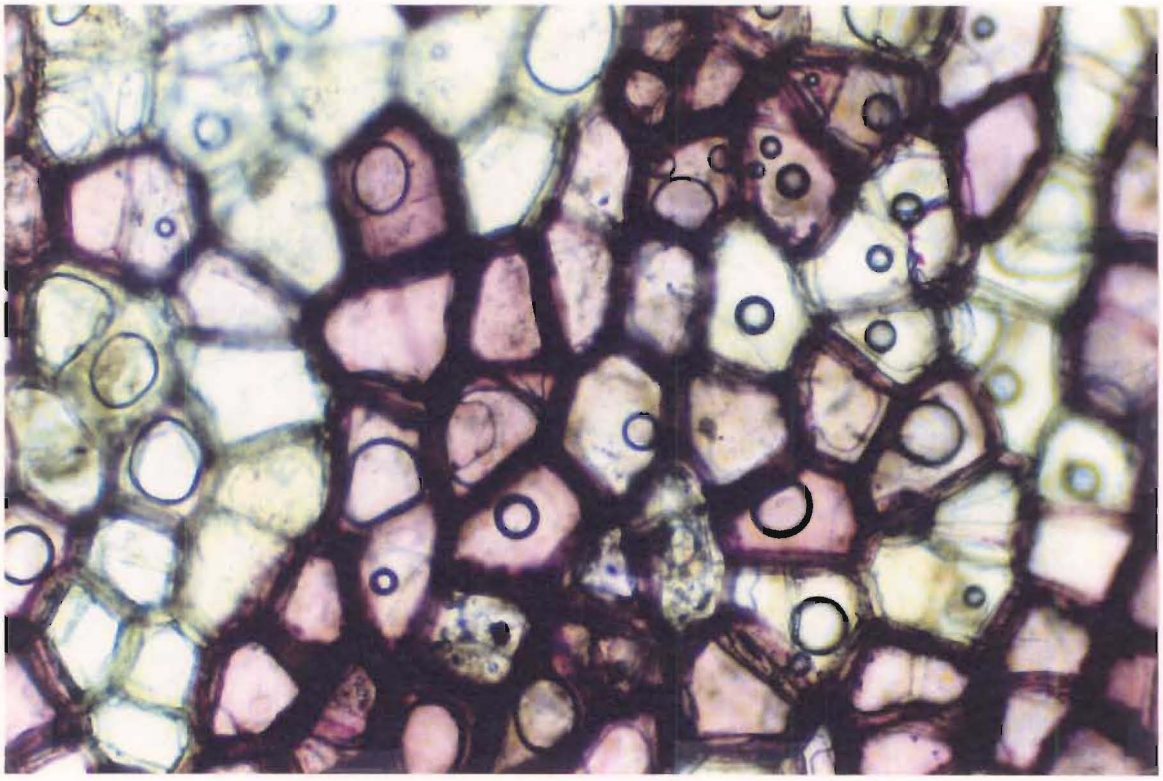
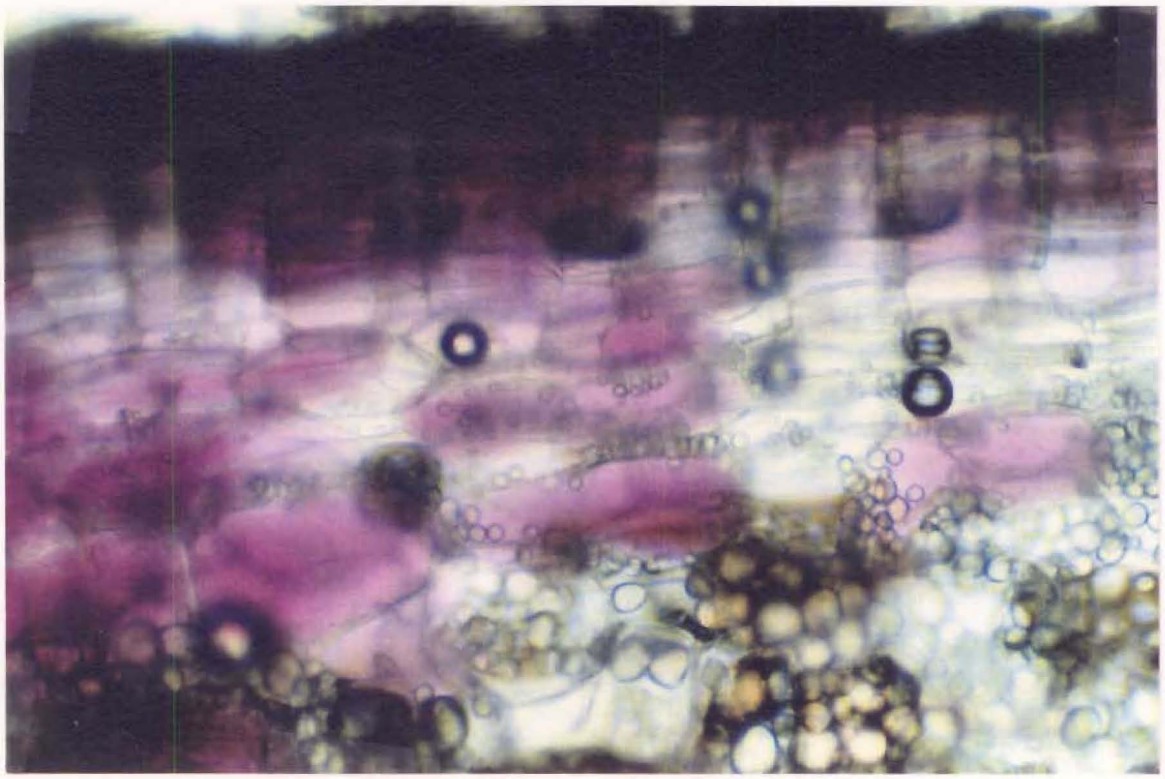
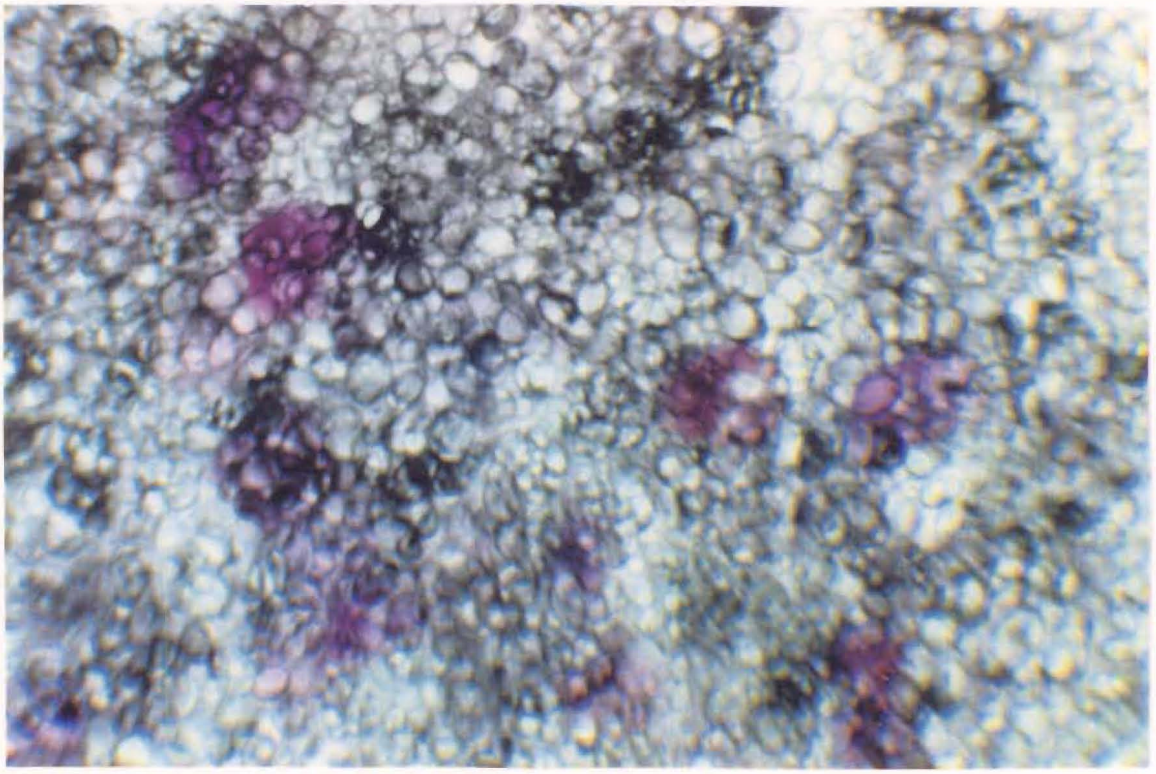


Figure 6.22 A typical tangential section of phellem from Urenika tubers.



_____ = 50μm

Figure 6.23 A typical transverse section from Urenika tubers.



_____ = 30μm

Figure 6.24 A typical transverse section of flesh from Urenika tubers.

6.4 Discussion

For this work to be reliable and meaningful, the sectioning technique was considered carefully. Sections of an even thickness were required, but this was not an easy task with a hand-held razor blade, especially as a number of tissues and cultivars were studied, having a range of tissue strengths. As well, the cell diameters also differed considerably between different tissues and different cultivars. Also, problems were encountered with the leaching of the pigment from the cut living cells, which made distribution studies difficult, and care had to be taken to compare cells within an undamaged layer, to ensure that a cell did not appear to be colourless and contain no anthocyanins simply because its contents had leached out, because of sectioning. To try and minimise these problems, relatively thick sections were cut, and the sections viewed in portions which were approximately four cells thick. In most cases, by changing the focus, a number of layers of each section were able to be viewed, to ensure that a representative undamaged layer was observed.

Others problems which may have been encountered were that 1) the cell contents of damaged cut cells may have leached out and affected the observed colour by altering the pH and changing the colour of the anthocyanins, 2) reactions of the anthocyanins with the starches and sugars may have taken place, as was shown to occur in Chapter 2, 3) the water used to mount the slides may have changed the pH surrounding the cells but, although some colour change of the leached anthocyanins may have occurred because of the higher pH of the water, this did not appear to affect the intact cells, and the excess colour of leached anthocyanins was removed, 4) plasmolysis may have been likely because water was used to mount the sections, although there was no evidence of this. It is more likely that water was absorbed by cells and this could have altered the natural cell conditions. Whilst there was no evidence of this, it might have, in hindsight, been better to have used isotonic, buffered washes for mounting the slides. On the other hand this might have caused problems in determining the optimum mounting solution for each cultivar.

In conclusion, taking these technical difficulties into account, and noting that this work was only carried out as a preliminary overview and not a complete study, these results do give some indications of the different strategies employed by the different cultivars which show such a wide range of colour intensities.

The differences of colour observed visually in the whole tuber, without magnification, appeared to be related to the concentration of anthocyanins (extracted in acetic acid in methanol, and concentration determined by HPLC, as in Chapter 3, and shown by the

data extracted from Appendix 3) (Table 6.1). This suggests that a key factor determining the visual colour of the tuber is the total concentration of anthocyanin produced (per gFW). However, the visual differences seen in the whole tuber could also be related to the distribution of anthocyanin observed at a cellular level. Here the difference in colour intensities of the red cultivars appeared to be due partially to a slight increase in the amount of anthocyanin produced in individual cortex cells, and possibly an increase in the proportion of cortical cells within a layer producing anthocyanin. However, the major factor appeared to be the amount of anthocyanin present in the phellem, because the dark red cultivar (Red Flesh) showed strongly coloured anthocyanin deposits around the cell walls of dead phellem cells, which was not evident in these cells of the lighter coloured red tubers (Red Rock, Desirée and Red Rascal).

Table 6.1 Anthocyanin concentration of selected cultivars
(data extracted from Appendix 3, Table A3.1)

Cultivar	Anthocyanin concentration (μg/gFW)	
	Skin	Flesh
Red Rocks	204.6	0
Red Rascal	1091.5	0
Desirée	1445.4	0
Red Flesh	3325.2	114.8
Tekau	81.8	0
Arran Victory	2386.9	78.2
Blue Derwent	3089.9	103.7
Stage II Blue	7446.6	218.5
Urenika	5778.8	1351.6

In general, purple tubers with a more intense colour had a greater proportion of more highly coloured cortical cells in tangential sections. They also had more layers of coloured cortical cells in the transverse view (*i.e.* the coloured layers of cortical cells went to a greater depth in tubers with a darker coloured skin). Additionally, as found in the red skinned tubers, the extra dark coloured tubers (Stage II Blue and Urenika) had intensely coloured deposits of anthocyanin around the cell walls of the phellem cells (diffuse in the cell walls, as well as the unattached "blobs").

There did not appear to be any change in the colour (λ_{max}) of the anthocyanin, for either the red or purple tubers, as the colour of the potato tuber became more intense, although it is doubted whether small changes in λ_{max} would be detected using these methods. The differences in colour observed in the extracellular "blobs" shown in Figure 6.4 and Figure 6.6 may be explained by the varying pH's outside the cells from the release of cellular contents from the cut cells, and the use of water as a mounting solution. The differences in colour of the cells in the vascular tissue, may also be explained in terms of pH differences, although in this case the purple/blue colours were probably caused by the more alkaline pH thought to occur in the intact phloem cells.

Phellem cells from Stage II Blue and Urenika showed a different pattern of anthocyanin location compared with similar tissues from other cultivars. In the other cultivars, the dead phellem cells either showed no colour, or showed the presence of deposits of residual anthocyanin pigment around the cell walls. The live cells of these other cultivars showed colour only in the lumen of the cell (*i.e.* the vacuole), although there were the occasional "blobs" of contaminating pigment which appeared to originate from the senescent or dead cells above the living cells that were being observed. However, in the live phellem cells from Stage II Blue and Urenika (Figure 6.22) the purple anthocyanin pigment was observed in the centre of the cells (in the vacuole) as well as in the cell walls. The reason for this is not known, but it is possible that the cells may have secreted the anthocyanins to the cell wall and/or they may have been senescent phellem cells and were observed in a transitionary state between living and dead. Flavonoids (usually aglycones) have been found to be secreted and linked to the cell wall (Strack *et al.*, 1989), however there is no mention in the literature of anthocyanin secretion to the cell wall, and the secretion of coloured anthocyanins appears to be unlikely. Typically only unglycosylated flavonoids (aglycones) are transported to the cell wall, because these are less polar than glycosylated flavonoids, and are absorbed better by the more non-polar cell wall. However, anthocyanidin aglycones are notoriously unstable and are likely to degrade. Although proanthocyanidins and condensed tannins (both polymeric anthocyanidin containing compounds) have been found attached to the cell wall, these compounds are colourless. Obviously this deserves further investigation.

The answer to the question of why some cells produce anthocyanin and other cells do not, is still unknown, but Lancaster *et al.* (1994) found a similar pattern in their studies of apple skin anthocyanins. The changes in colour of single cells may be caused by an internal concentration effect. It is known that the self-association of anthocyanins occurs at high concentrations, especially in anthocyanins with sugars at both 3- and 5-positions (Harborne, 1988), and that this is a copigmentation effect with an increase in absorbance

and a bathochromic shift in λ_{max} (Section 2.1.1.3). Changes in the cellular concentrations of sugars and starches may also have some effect on the colour observed.

Further study of the differences at the cellular level would be useful to answer this and other questions. In particular, the use of image analysis to quantify the number and intensity of coloured cells, and also the use of a microspectrophotometer to measure the different λ_{max} values observed in the different coloured cells would be useful. Additionally, the comparison of colour intensity values with the anthocyanin concentration values could be utilised to determine what relationship is present between these two variables, and whether the anthocyanin colour of different cultivars is affected in different ways by pH or copigmentation.

CHAPTER 7

Effect of cooking on potato tuber anthocyanins

7.1 Introduction

One of the reasons for this study of the effect of cooking on the tuber anthocyanins is an anticipated increase in consumer demand for coloured potatoes, particularly those with coloured flesh for use in attractive looking potato salads (Figure 7.1) and novelty crisps (Figure 7.2). Therefore, the stability of anthocyanins and the maintenance of colour during various cooking and crisping practices becomes of key importance and was investigated here.

Although extensive research has been conducted on the cooking and crisping properties of potato tubers, no reference to studies of this nature on potato anthocyanins was found in the literature. A number of studies have been carried out recently to investigate the factors which determine the texture of tubers during cooking (Andersson *et al.*, 1994; Quinn and Schafer, 1994; van Marle *et al.*, 1994), the effect of frying on tubers, particularly with respect to the browning of crisps and fries caused by the Maillard reaction (Coles *et al.*, 1993; Khanbari and Thompson, 1993; Shock *et al.*, 1994), and the changes in the oil during the frying of crisps with respect to the crisp quality (Hebash and Fadel, 1994).

Anthocyanins are known to degrade at high temperatures, and this topic has been discussed previously in Chapter 2. In addition, Daravingas and Cain (1968) investigated the thermal degradation of anthocyanins in black raspberries and found that thermal degradation was reduced at lower pH's, and that the replacement of the O_2 atmosphere with N_2 enhanced pigment stability, whilst the addition of sugars, and in particular sugar degradation products, increased anthocyanin breakdown.

Figure 7.1 Potato salad using white (Rua) and purple (Urenika) potatoes.

Figure 7.2 Urenika crisps after frying for 90s in vegetable oil at 180°C.

7.2 Methods and Results

Colour was measured with the image analysis system as in Chapter 4, and all cooking experiments were carried out with Urenika tubers field grown at Lincoln. All tubers used in these experiments were approximately 100g.

7.2.1 Effect of washing tuber slices

It was observed that when cut tuber tissue was placed in water, either for the washing of crisps before frying, or tubers for boiling, some anthocyanin leached out of the tissue and the water became purple coloured. Therefore, the loss of colour after washing tuber slices was investigated. Colour density of tuber slices was measured immediately after cutting, and the tuber slice then washed in cold water, and the colour density measured again. When this was carried out for thirty slices it was found that the average colour loss was less than 2%.

7.2.2 Steaming

Unpeeled tubers were steamed in a commercial steamer for up to three hours, and then left to cool for approximately one hour. The still warm tubers were sliced into five slices and the colour density of each slice determined using ten tubers for each cooking time. Uncooked tubers were used as controls (time 0), and the colour density of five slices for each of ten tubers was similarly measured. When the anthocyanin colour was measured after up to two hours steaming there was a slight decrease in colour, although these differences were not significant (Figure 7.3). After three hours there was a significant, visible degradation of the anthocyanins, with tubers having a lighter grey/purple colour instead of dark purple, and a 46% loss of colour was recorded. However, after this length of time the potatoes were vastly over cooked and only held together by the surrounding skin. In practice, little or no colour would be lost by cooking the tubers by this method, because the usual cooking time for steamed potatoes is 40-50min.

7.2.3 Boiling

7.2.3.1 Cubes

Tubers were cut into ~1cm^3 cubes, which were washed and their colour measured, before cooking. At least seven cubes from each treatment were cooked, one at a time, in boiling water for 2, 4, 6 or 8min, after which the colour of the surfaces of the cubes was re-measured. The immersion of the cut cubes into boiling water caused the anthocyanin to

leach into the water, and the water turned a strong purple colour. This purple colour in the water appeared to remain stable for 10-15min, but after this period the anthocyanin degraded and the purple was replaced by a green colour after 25-30min of boiling. Cubes were cooked sufficiently to be edible after 2-4min and, after 6min had begun to crumble. A 41% loss of colour had occurred after 2min boiling which increased to 64% after 8min (Table 7.1).

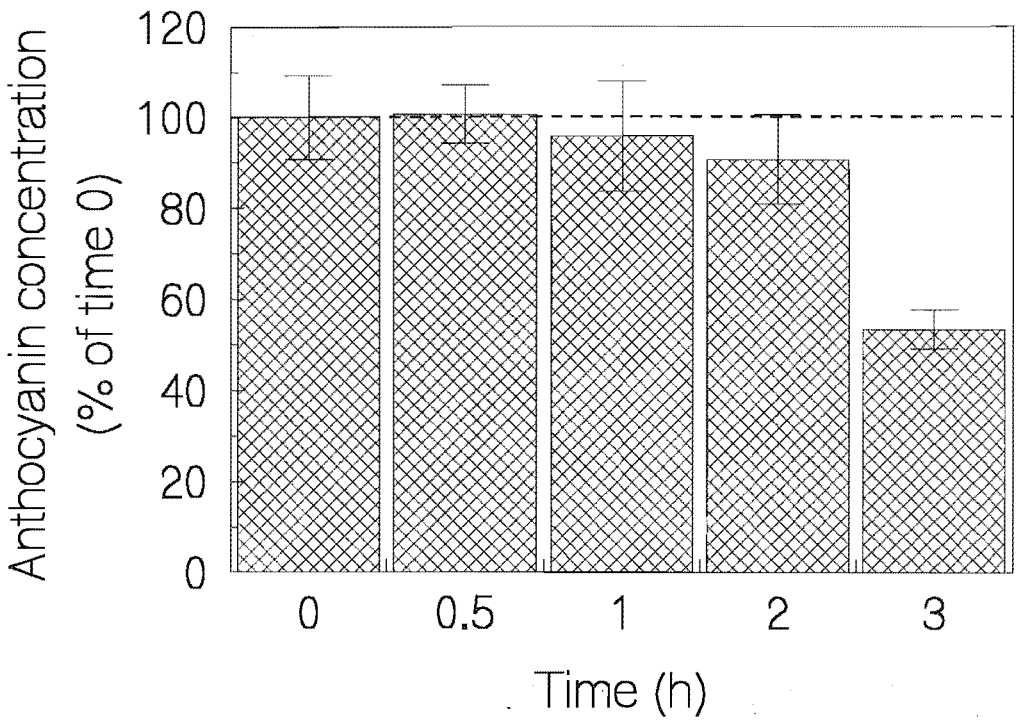


Figure 7.3 Amount of anthocyanin colour remaining after whole tubers were steamed. Error bars represent ± 1 standard error.

Table 7.1 Amount of colour lost from the surface of $1cm^3$ cubes after boiling.

Time (min)	loss of colour (%)
0	0
2	40.9
4	41.8
6	55.1
8	64.0

7.2.3.2 Whole tubers

Tubers were boiled whole, either peeled or unpeeled, with each treatment being cooked in separate pans of boiling water with five tubers taken out at 5 or 10min intervals for analysis. Each tuber was sliced into five equal slices and the colour density determined for each slice.

a) Peeled tubers

Peeled tubers were ready to eat after 5-10min however, after 10min some sloughing was observed on the outside of the tuber. After 15min tubers were extremely soft and after 20min tubers had completely disintegrated so no further readings could be taken. No decrease in colour was found up to 10min boiling of peeled tubers, but at 15min a 30% loss in colour had occurred (Figure 7.4).

b) Unpeeled tubers

Because these tubers still had the skin intact, they were able to be boiled for a longer time than peeled tubers without falling apart. There was a small decrease in the colour of slices of unpeeled tubers after 20min boiling, although this was barely significant, however a 30% loss of colour had occurred after 40min (Figure 7.5). The anthocyanin pigments were also extracted and measured spectrophotometrically (as in Section 4.2.2), and results showed a similar pattern to those shown in Figure 7.5.

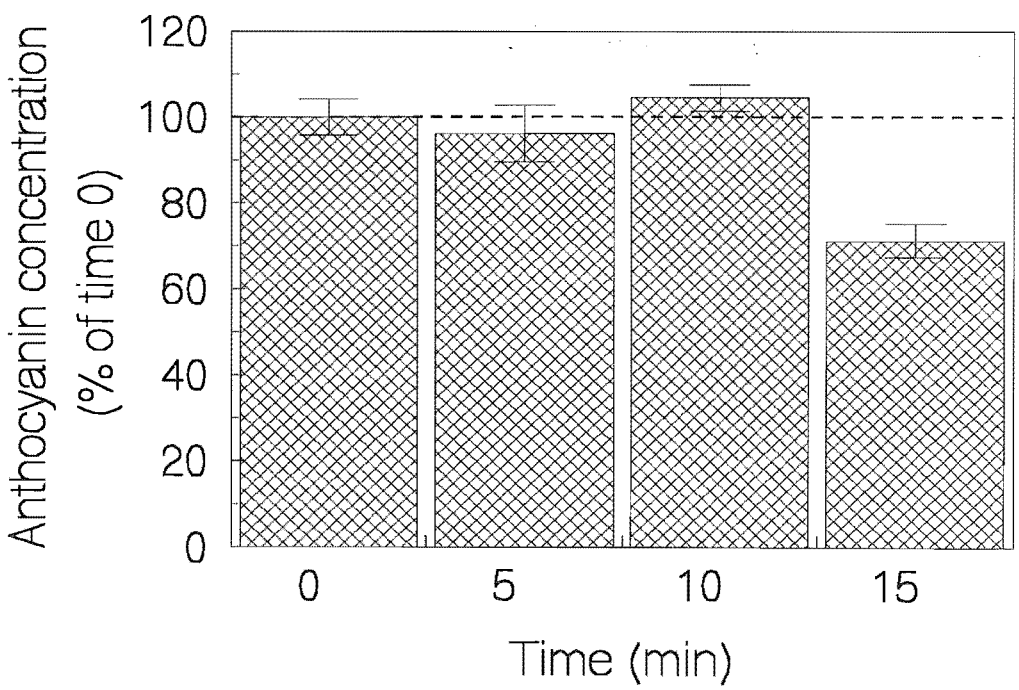


Figure 7.4 Amount of colour lost from slices after whole peeled tubers were boiled. Error bars represent ± 1 standard error.

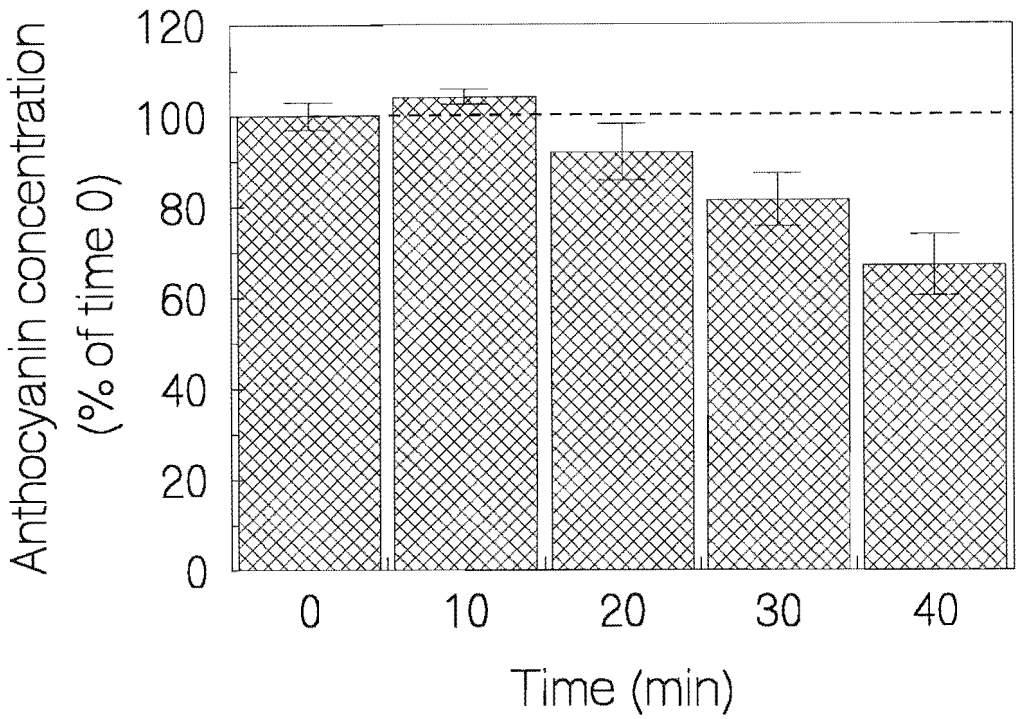


Figure 7.5 Amount of colour lost from slices after whole un-peeled tubers were boiled. Error bars represent ± 1 standard error.

7.2.4 Crisping

Crisps were sliced from the centre of tubers with a hand held crisp slicer to give slices with an even thickness of 1.5mm. Slices were washed in cold water, excess water removed, and their colour density measured. Crisps were then fried in vegetable oil at 180°C, for up to 120s, in flat wire baskets so that each crisp could be identified, and the colour measured again after cooking. Crisps were cooked and ready to eat after 60-90s.

It was found that image analysis was not appropriate for the measurement of anthocyanin colour in crisps, because after 120s the crisps had turned pale brown, and an increase in colour density was measured, caused by this brown colour. It can be seen in Figure 7.2 that, although the crisps did go brown, the purple anthocyanin colour appeared stable. Attempts were made to extract anthocyanins in 15% acetic acid in methanol, and measure their concentration, however this proved unsuccessful because only a low proportion of the total anthocyanins were extracted, probably because of the penetration of the oil throughout the tissue.

7.3 Discussion

Although anthocyanins have been found to degrade at high temperatures (reviewed in Markakis, 1974; Francis, 1989), typically this did not occur during the normal cooking time of the potato, whether boiled, steamed or fried. For retention of anthocyanin colour, steaming was found to be a very effective way of cooking tubers, with no loss of anthocyanin during the normal cooking time and, because there was no free water, there was no loss of anthocyanin by leaching. However, tubers took longer to cook by steaming than boiling, which may be a significant disadvantage to many consumers.

Boiled whole tubers, either peeled or unpeeled also retained all of their colour, whereas small boiled cubes lost 40% of their colour by the time the cubes were ready to eat. Most of the colour lost from these small cubes was lost within the first two minutes (Table 7.1). It is suggested that this is caused by the leaching of anthocyanin pigments into the boiling water from the cut surface of the tuber, rather than degradation of the anthocyanin, because only the colour of the cut surface was measured before and after cooking (not the inside of the cube). This was supported by the observation that the centre of these cubes still retained their purple colour. The leaching of colour was not surprising given that these surface cells were likely to have been cut open. This 40% loss from the surface of the boiled cubes was much higher than the 2% of colour lost when slices were washed in cold water, and thus the higher temperature of the boiling water appeared to increase the rate of leaching into the water, possibly by further degradation of cells at the higher temperatures. Therefore, it is suggested that in the preparation of purple potato salads, the tubers should be cooked whole, and cubed after cooking to retain their full purple colour. The peeled tubers also showed a more rapid reduction in colour density than the unpeeled tubers when boiled. It is hypothesised that the tuber skin prevented the more rapid loss of colour by reducing the leaching of anthocyanins into the water.

The purple colour was also retained when potato slices were fried, and the preparation of purple crisps was achieved, even though the loss of colour, if any, could not be quantified.

In summary, the anthocyanins present in Urenika tubers were stable, and did not suffer thermal degradation in the time period required for potatoes to be cooked by steaming, boiling or crisping. However, some degradation did occur in boiled and steamed potatoes after three to four times the length of time required for cooking.

Image analysis appeared to be an appropriate tool for the measurement of colour in tubers, and has been used previously by workers for the measurement of colour in potato crisps (Coles *et al.*, 1993; Scanlon *et al.*, 1994). The colour density obtained by image analysis was correlated with the anthocyanin concentration measured by extraction and spectrophotometric analysis. Image analysis proved to be superior for these experiments because it was far less time consuming, and was non-destructive, so that changes in a particular sample could be measured before and after treatment. This was particularly useful in measuring changes occurring in cubed tubers, because it meant that the same sample could be measured before and after boiling, which eliminated the high variation between samples, and the necessity for larger sample sizes. Limitations in the use of image analysis were found when other colour changes also occurred, as when the fried (crisped) slices showed an increase in brown colour which was measured as an increase in density.

This work has been submitted for publication (Lewis *et al.*, 1996).

CHAPTER 8

Concluding discussion

8.1 Biosynthesis of anthocyanins

8.1.1 Anthocyanins and hydroxylation patterns

Anthocyanins, flavonoids and phenolic acids were surveyed in tetraploid (*S. tuberosum*) potato cultivars and diploid *Solanum* species. The concentrations of these compounds were related to the colour of the tubers and to regulation of anthocyanin biosynthesis. This is the first time that a biochemical analysis of these three classes of compounds has been carried out in potato tubers together in one study.

Most commercial potato cultivars have white tubers because in the 1800's and early 1900's there was selection for tubers with white skins and white flesh (*i.e.* no anthocyanins and low concentrations of carotenoids). However, there are many cultivars with coloured (anthocyanin containing) skin and/or flesh, and these tend to be older cultivars which have not been through plant breeding programs for selection of attributes such as disease resistance, tubers with shallow eyes, uniform tuber size, etc.

Pink, red and purple potato colours result from anthocyanins in the skin and/or flesh of the tuber. The presence of anthocyanin aglycones in different coloured tubers is shown in Table 8.1. The pink/red coloured tubers tended to contain mostly the monohydroxylated Pg-glycosides, with lower concentrations of the dihydroxylated/ methylated Pn-glycosides. Purple tubers generally contained only trihydroxylated/ methylated anthocyanins. Light to medium purple coloured tubers contained mostly Pt-glycosides with low concentrations of Mv-glycosides whilst the dark purple/black tubers contained similar concentrations of Pt-glycosides, plus very high concentrations of Mv-glycosides (Chapter 3). No Cy-glycosides were found in the tubers, although some flowers contained low concentrations of Cy-3-rut.

The predominant glycosidic group was 3-(*p*-coumaroyl-rutinoside)-5-glucoside, with lower amounts of 3-rutinoside. Although only these two glycosidic patterns have been reported previously (Harborne, 1960a: Simmonds and Harborne, 1965), the presence of minor anthocyanins containing other different glycosidic patterns was reported here

(Chapter 3), although the identities of these were not determined. The cultivars with red tubers may have economic importance for production of natural food colourants, especially cultivars such as Red Flesh which have high concentrations of Pg-glycosides in the skin and flesh, and produce abundant quantities of large tubers. Cultivars with purple tubers may also be useful for food colourant production, however grapes are currently used for production of natural purple colourants.

Table 8.1 Anthocyanin aglycones present in tubers.

Tuber colour	Anthocyanin concentration (μg/gFW)					
Hydroxyl pattern	OH	2OH		3OH		
Aglycone	Pg	Cy	Pn	Dp	Pt	Mv
White	-	-	-	(-)	(-)	(-)
Pink/red	***	-	*	-	-	-
Light purple	-	-	-	*	**	*
Dark purple	-	-	-	*	**	***

-	generally absent	Pg	pelargonidin
(-)	sometimes present in small quantities	Cy	cyanidin
*	50-500μg/gFW	Pn	peonidin
**	500-2000μg/gFW	Dp	delphinidin
***	2000-6000μg/gFW	Pt	petunidin
OH	monohydroxylated B-ring	Mv	malvidin
2OH	dihydroxylated B-ring		
3OH	trihydroxylated B-ring		

The concentrations of flavonoids and phenolic acids were related to the anthocyanin concentration in tubers of different colour classes. Tubers generally had very low concentrations of flavonoids, with coloured tubers (red and purple) having two to three times the concentration of that in white tubers. Most of the flavonoids were the dihydroxylated Qu-glycosides, with similar concentrations of these found in red and purple tubers. Red tubers contained higher concentrations of the monohydroxylated flavonoid, Km-3-rut, than that found in white or purple tubers (Chapter 3). This was associated with the presence of the monohydroxylated anthocyanins, Pg-glycosides, which were found only in red coloured tubers. Unfortunately, the concentrations of the trihydroxylated flavonoid, My, could not be determined in coloured tubers, owing to its co-elution with the major anthocyanins present.

Phenolic acids were present in high concentrations in tubers, but there appeared to be no relationship between the hydroxyl pattern of the phenolic acids with that of the anthocyanins in red or purple tubers. However, the concentration of phenolic acids in coloured tubers was twice that in white tubers.

8.1.2 Determination of tuber colour

The main factor influencing the colour of the tuber appeared to be the actual concentration of the anthocyanin itself, although other factors may have been important. During studies of the cellular structure and anthocyanin location in the tuber, it was found that the colour intensity of strongly coloured tubers was caused by a higher proportion of coloured cells within each layer of cortical cells and a greater number of layers of cortical cells containing anthocyanins (Chapter 6). Additionally, the very strongly coloured cultivars (Stage II Blue and Urenika) had intensely coloured deposits of anthocyanins around the cell walls of both the dead and live phellem cells. It was interesting that the anthocyanin pigment occurred in the cell walls as well as in the vacuole where it is typically found. The anthocyanin in the cells from phloem tissues showed a bluer colour than cells from periderm tissues, and it was proposed that this was caused by the more alkaline pH found in phloem cells.

Potato tubers contained high concentrations of phenolic acids (Chapter 3), which are known to act as copigments and increase the intensity and alter the colour of anthocyanins (Chapter 2). The flavonoids (flavonols, flavan-3-ols, and flavanones) may also have influenced the anthocyanin colour by copigmentation, however in tubers they occurred in much lower concentrations than the phenolic acids. No work was carried out on the copigmentation effect of phenolic acids or flavonoids, and this may be a useful topic to follow up. Carbohydrates, as well as influencing the biosynthesis of anthocyanins, were also found to affect the colour of the anthocyanin molecule itself, although this was not actually a copigmentation effect because no change in λ_{max} occurred (Chapter 2). Starches (amylose and amylopectin) with their helical structures, and other carbohydrates which formed inclusion complexes (cyclodextrins) decreased the intensity of the anthocyanin colour. In contrast, sugars (glucose, maltose and sucrose) increased the colour intensity shown by anthocyanins. These effects may be important in determining the colour of the final potato product, especially in cooked potatoes when the cellular structure is altered, because the tuber contains large quantities of starch, and also variable concentrations of sugars, which may alter the anthocyanin and tuber colour.

8.2 The life of a coloured potato

An important part of this thesis was the study of coloured tubers as a food product. Assuming that the consumer wants an intensely coloured tuber for use in products such as novelty coloured potato chips or coloured potato salads, any factor which influences the colour of the end product is important. Therefore, the tuber and its colour was followed from tuberization to the final product.

Concentration, rather than amount, of anthocyanins in tubers was measured because concentration rather than the amount of anthocyanin per tuber *per se*, is important in determining the intensity of colour. Newly initiated tubers typically started off with no colour, and the rate of anthocyanin production of developing tubers depended on the cultivar, with tubers that contained a higher final anthocyanin concentration having more rapid production of anthocyanins in the developing tuber (Chapter 4). As the size of the tuber increased, so did the anthocyanin concentration, up to a maximum at a certain tuber size, which depended on the cultivar, after which the concentration remained constant, or often decreased in the larger tubers. The concentration of anthocyanins at the stem end of the tuber remained higher than at the bud end until the maximum anthocyanin concentration for that cultivar was reached, when concentrations became similar in both ends. The timing of harvest (with respect to tuber size) is important to enable tubers with a maximum anthocyanin concentration (*i.e.* colour) to be harvested. Environmental conditions and mineral, nutrient and water availabilities may also affect the anthocyanin concentration, but were not studied here. However, in general, environmental and nutritional stress tends to induce an increased production of anthocyanins.

Although anthocyanins, when present, contribute the major colour in potato tubers, carotenoids may also play a significant role in tuber colour, especially in the flesh of tubers when anthocyanins are absent. The concentration of carotenoids was highest in small tubers (<10g) and decreased as the tuber increased in weight. This change was the opposite pattern to that which occurred for anthocyanin biosynthesis.

Storage conditions were also important in the final colour of the tuber, and cold storage (4°C) was found to increase the anthocyanin concentration, whilst storage at warmer temperatures (above 10°C) resulted in a small loss of anthocyanins (Section 4.4.3.3). Therefore, the usual storage conditions for potato tubers (at about 4°C to reduce sprout growth and disease) are also ideal for maximum anthocyanin colour. The increase in anthocyanins in cold storage appeared to be related to carbohydrate metabolism because the sugar concentration in tubers is found to increase after cold storage caused by the

degradation of starch. Additionally, there was a change in the distribution of the anthocyanin within the tuber during cold storage, with higher concentrations of anthocyanins found towards the bud end than the stem end after storage (instead of higher concentrations at the stem end, as was found in developing tubers). This increased concentration at the bud end is also thought to be associated with carbohydrate metabolism, and the increase of sugars found at the bud end due to sprout formation (Chapter 4).

For the consumer it is important that highly coloured tubers retain their colour during cooking, and that the final product is coloured. It was found that during normal cooking (boiling, steaming or crisping), there was no thermal degradation of anthocyanins, and intensely coloured products were produced (Chapter 7). Although some loss of colour was found after two to three times the normal length of cooking time, these tubers were vastly overcooked. The only significant loss of anthocyanin when potatoes were cooked for the normal length of time was caused by leaching from cut tissues when boiling, and therefore coloured tubers should be cooked whole and cut up after cooking (e.g. for potato salads) for maximum retention of colour.

8.3 Expression of different pathways

The concentrations of flavonoids and phenolic acids were found to increase in diseased tubers infected with *Phytophthora infestans* (Chapter 3.5.4). In particular, some flavonoids were found in diseased tubers which were not detected in healthy tubers. This suggested that the expression of flavonoid pathways in tubers was altered by disease.

There appeared to be differential expression of anthocyanins, flavonoids and phenolic acids in tubers, and also different expression of these classes of compounds between tubers and the flowers and leaves. Tubers contained low concentrations of flavonoids (other than anthocyanins) (200-300μg/gFW) and high concentrations of phenolic acids (2000-5000μg/gFW), whilst flowers and leaves contained high concentrations of flavonoids (1000-3000μg/gFW) and low concentrations of phenolic acids (400-1100μg/gFW). In coloured tubers, the phenylpropanoid pathway through to the phenolic acids and also the anthocyanin pathway were both very active producing high concentrations of phenolic acids and anthocyanins. However, the other flavonoid pathways (e.g. flavonol, flavone) appeared to have no, or very low, through-put. Therefore, in tubers there appeared to be a block in all the flavonoid pathways (except anthocyanins). The expression of these pathways in flowers and leaves was different from that shown by tubers, because in flowers and leaves the concentration of flavonoids (other than

anthocyanins) was high, and the concentration of phenolic acids relatively low. There were also differences between species in the production of some flavonoids in the flowers and leaves. The total concentration of flavonoids was similar in most cases, but the composition varied, with most of the *S. tuberosum* cultivars having high concentrations of Qu-glycosides, whilst most of the imported *Solanum* species had low concentrations of the Qu-glycosides, except rutin which remained relatively high. Of the imported species which had high concentrations of the Qu-glycosides, two of these (*S. stenotomum* and *S. sparsipilum*) are believed to be ancestors to *S. tuberosum*. There were other significant differences between *S. tuberosum* cultivars and the other imported *Solanum* species. *S. tuberosum* flowers typically contained lower concentrations of flavonoids and phenolic acids, whilst tubers and leaves contained higher concentrations of flavonoids, compared with other *Solanum* species.

The anthocyanin, flavonoid and phenolic acid pathways in tubers also showed differential expression after exposure to light. In experiments carried out with foil-covered minitubers (*i.e.* minitubers in the dark), there was no anthocyanin present in foil-covered minitubers before the leaves were exposed to light, but after light exposure there was a huge increase in anthocyanin concentration (Section 5.4.9). The flavonoids showed a three-fold increase after light exposure, but in contrast to anthocyanins and flavonoids, the phenolic acids showed no change in concentration in minitubers after the leaves were exposed to light. Therefore, these three pathways in the tuber all showed different responses in expression after exposure of the leaves to light.

It appears that there may be the potential for all parts of the pathways to be active, but with different expression and regulation. This suggests that instead of one pathway with branches, there may be parallel pathways, for example, the anthocyanin, flavonol and flavone pathways operate in parallel pathways.

8.4 Effect of light

Anthocyanin production in coloured tubers, and also other red or purple vegetables formed underground, for example radishes, occurs completely in the dark. This is surprising and unusual because most plants and plant organs require direct light for the production of anthocyanins; most flowers require light for their colour to become visible, and many fruits require light for the expression of red colour associated with ripening. For example, if apples which usually ripen to a red colour are covered and kept in darkness, they will remain a yellow or green colour with no production of the red anthocyanins usually associated with ripening. It is even possible to expose only a small

portion of the apple to light, with the remainder in the dark, and then anthocyanin production occurs only in the area exposed to light.

At the start of this thesis it was hypothesised that potato tubers were unusual and different from these other plants and plant organs, and could produce anthocyanins without the requirement of light. Therefore, a major aim of this thesis was to investigate the question of "how do potato tubers produce anthocyanin in the dark?" It was hoped that the answer(s) to this question may aid in the establishment of genetic manipulation techniques to enable the production of anthocyanins in flowers and fruit independent of light. However, it was found that whilst tubers of some cultivars (e.g. RKE and Urenika) could produce anthocyanin with no light, others (e.g. Desirée) produced none, and in all cultivars the exposure of the plant to light resulted in the increased production of anthocyanins (Chapter 5). Therefore, although tubers do produce anthocyanin in the dark they are similar to all other plants in that exposure to light increases the production of anthocyanins. Consequently, the questions were re-directed towards "how do the tubers detect that the aerial parts of the plant are exposed to light?", "what effector molecule(s), if any, are transported from the leaves to the tubers?", "what are the effects of different light intensities and wavelengths on anthocyanin production in the tubers?".

8.4.1 Is a carbohydrate or "trigger" compound responsible?

It was confirmed that in the field no light reached the growing tubers, so that anthocyanin production in tubers occurred in total darkness (assuming that the anthocyanins themselves were not transported to the tubers from the photosynthetic parts of the plant). The experiments carried out during the course of this thesis (mainly using minitubers as experimental tools) indicated that light exposure of the aerial parts of the plant was either essential for, or vastly increased, the production of anthocyanins in tubers (Chapter 5). This was confirmed by other experiments, for example, after the light exposure of plantlets with foil-covered minitubers (*i.e.* minitubers in the dark attached to the plantlet), the anthocyanin concentration in the covered minitubers increased, almost to the concentration of minitubers kept uncovered and in the light (Section 5.4.9). It was also found that, in dark grown minitubers removed from the plantlet and exposed to light, anthocyanin production was limited (Section 5.4.8). Therefore, the first important conclusion was that light was important in anthocyanin biosynthesis in potato tubers, and that it was the exposure of the aerial parts of the plant (*i.e.* leaves and stem) that was important, not just the exposure of the tubers to light. Hence, potatoes appear to be different from other plants, especially when compared with fruit where, for example in apples, direct light exposure of the fruit is necessary for the synthesis of anthocyanins, whereas in potato tubers direct light on the tuber is not

required, but indirect light is necessary for maximum anthocyanin production. For Desirée minitubers, there appeared to be a maximum anthocyanin concentration (about 3.2ng/cm^{2}SA) attainable, and exposure of the tuber, as well as the leaves, to light only increased the rate of anthocyanin synthesis, not the final anthocyanin concentration (Chapter 5).

So, how does the tuber know that the aerial parts of the plant have been exposed to light? The results indicate that there was transport of some signal molecule from the light exposed parts to the tubers after light exposure (Chapter 5). It was thought that this signal may be a carbohydrate or a "trigger" compound. Transport of carbohydrates from the leaves to the tubers is known to occur, and provide "food" for the roots, rhizomes and growing tubers but, was carbohydrate responsible for inducing anthocyanin biosynthesis? From experiments carried out during the course of this thesis, it was proposed that an adequate supply of carbohydrate to the tuber was required for production of anthocyanins. It appeared that carbohydrates were used in the growth of the photosynthetic parts of the plant first, then in the production of tubers and, only if there was sufficient carbohydrate remaining, was it used for secondary metabolism and the production of phenolic acids, flavonoids and anthocyanins. However, it seemed that an adequate carbohydrate supply was not all that was necessary for the production of anthocyanins because, if Desirée minitubers were kept in the dark, no anthocyanin was produced even though excess sucrose was supplied in the medium. Thus, exposure of these plantlets to light was necessary for anthocyanin synthesis and to initiate the production and/or transport of a "trigger" molecule to the tubers where, if there was sufficient carbohydrate, anthocyanin production began. This hypothesis of transport of molecules (carbohydrate and "trigger") from the leaves to the tubers is supported by the fact that, in both field grown tubers and minitubers, anthocyanin production began in that part of the tuber closest to the stem (stem end), and spread gradually towards the bud end.

Why are the tubers of only some cultivars coloured, whilst tubers of many other cultivars remain white? Experiments with grafted potatoes (Section 5.4.10) suggested that only tubers which were genetically competent were able to produce anthocyanins. It was not just the presence of the effector molecule(s) which determined whether or not anthocyanin was produced. Therefore, from the results of the grafting experiments, it was hypothesised that, for a tuber to be coloured it must 1) have the genetic capability for anthocyanin production in the tubers (which may involve the recognition of the "trigger" molecule), 2) produce and/or transport a "trigger" molecule from the leaves to the tubers and, 3) have adequate carbohydrate left over from primary metabolism for secondary metabolism and the production of anthocyanins, with both the production of

the "trigger" compound and carbohydrates being light dependent. This was confirmed by results from Chapter 3, where regulation was thought to be controlled by substrate limitation. However, this hypothesis of genetic capability is confused by the work of Brown and Riley (1976) who found that during the normal growth of "white" skinned tubers no anthocyanin was produced, but after harvest the direct exposure to light of tubers resulted in anthocyanin production. This suggests that these "white" skinned tubers did have the genetic capability to produce anthocyanin, but why was no anthocyanin produced under normal growth? There are a number of possibilities; 1) no "trigger" was produced, however this has been proved unlikely by the grafting experiments or, 2) the "trigger" was not recognised by the tuber so anthocyanin production was not induced, and the direct exposure of the tissue to light induced a small amount of enzyme activity directly, without the need for a "trigger". This is supported by results with minitubers (Section 5.4.8) where minitubers removed from the plant and exposed to light produced small quantities of anthocyanin, whilst higher anthocyanin production resulted when the minituber remained attached to the light exposed plant. This suggests that" direct light to the tuber results in small amounts of enzyme activities and anthocyanin production, whilst indirect light and the proposed transport of "trigger" compound results in higher enzyme activities and anthocyanin production. It also suggests that all cultivars contain the genes for anthocyanin production (but perhaps not the ability to recognise the "trigger" compound). This is backed up by results from Section 3.5.4 where diseased tubers contained flavonoids not present in healthy tubers, so in healthy tubers the genes were present but not active. The different responses of different cultivars suggest that there may be varying threshold for the "trigger" compound in different cultivars.

The production of anthocyanins after light exposure appeared to be a high irradiance response (HIR) because short bursts of light had no effect on anthocyanin production, and up to eight hours of light was required before there was significant anthocyanin synthesis (Section 5.4.6). Anthocyanin production was induced by red, blue and purple light, which suggests that this response was mediated by phytochrome and cryptochrome (Section 5.4.5). Exposure of dark grown tissue-cultured plantlets and minitubers to light resulted in the increased activity of the whole secondary pathway involved with anthocyanins, and increased the final concentrations of phenolic acids, flavonoids and anthocyanins. All the enzymes tested (PAL, C4H, CHI, F3H, F3'H, DHR and GT) showed increased activity after light exposure (Section 5.4.7). It would be interesting to determine whether the enzyme activities of covered minitubers (in the dark) with the plantlet in the light, showed similar enzyme patterns. That is, to determine whether it was the direct effect of light on the tubers which caused these increases in enzyme activities or whether the increases were caused by the "trigger" molecule. More work

needs to be carried out to determine the effects of different wavelengths and different light intensities on the biosynthesis of anthocyanins in potato tubers, and the changes in enzyme activities and mRNA levels.

In conclusion, anthocyanin biosynthesis in potato tubers has a different regulation of colour compared with fruit and flowers in general, and even the flowers and leaves of the potato plant. Although direct light on to the tuber is not required for production of anthocyanins, as it is in many fruit, exposure of the leaves to light is necessary for maximum synthesis. Therefore, the shoot is important in mediating anthocyanin colour in tubers, probably via a "trigger" compound transported to the tubers, although an adequate supply of carbohydrate is also necessary. There appeared to be different thresholds for the "trigger" compound in different cultivars.

ACKNOWLEDGEMENTS

There are so many people who have helped in some way during the preparation of this thesis and I would firstly like to thank all those that I am unable to mention personally here.

The financial support for this work was supplied by the NZ Institute for Crop & Food Research Ltd and I am indebted to them for the chance to complete this thesis. The provision of research facilities was supplied by the University Grants Committee and the University of Canterbury.

I am very grateful to my supervisors, Dr John R.L. Walker and Mrs Jane E. Lancaster, and to Dr Tony J. Conner, for their support and guidance during this research. A special thanks goes to these people and others who have helped or given technical assistance, especially:- Dr Kevin Sutton for HPLC and helpful discussions, Mrs Maggie Tisch for all her support and running the lab so efficiently, Mr Graham Young for assistance with computers, Mr Bruce Clark for MS analysis of anthocyanins, Dr John Blunt for nmr analysis of anthocyanins, Mr Dougal Holmes for photography, Dr John McCallum for help with programming the image analyser and Dr Peter Meredith for helpful discussions.

In particular, a special thanks to Dr Peter Jarvis for the many, many hours he spent reading over this thesis, and to Greg and the rest of my family and friends for their support and encouragement over the years and during the course of this thesis.

REFERENCES

Akita, M., Takayama, S., 1994a. Stimulation of potato (*Solanum tuberosum* L.) tuberization by semicontinuous liquid medium surface level control. *Plant Cell Reports*, **13(3-4)**: 184-187.

Akita, M., Takayama, S., 1994b. Induction and development of potato tubers in a jar fermentor. *Plant Cell, Tissue and Organ Culture*, **36(2)**: 177-182.

Alchanatis, V., Peleg, K., Ziv, M., 1994. Morphological control and mensuration of potato plantlets from tissue cultures for automated micropropagation. *Plant Cell, Tissue and Organ Culture*, **36(3)**: 331-338.

Andersson, A., Gekas, V., Lind, I., Oliveira, F., Öste, R., 1994. Effect of preheating on potato texture. *Critical Reviews in Food Science and Nutrition*, **34(3)**: 229-251.

Arakawa, O., 1988. Photoregulation of anthocyanin synthesis in apple fruit under UV-B and red light. *Plant Cell and Physiology*, **29(8)**: 1385-1389.

Asen, S., Stewart, R.N., Norris, K.H., 1972. Co-pigmentation of anthocyanins in plant tissues and its effect on colour. *Phytochemistry* **11**: 1139-1144.

Baruah, P., Swain, T., 1959. The action of potato phenolase on flavonoid compounds. *Journal of the Science of Food and Agriculture*, **10**: 125-129.

Bassim, T.A.H., Pecket, R.C., 1975. The effect of membrane stabilizers on phytochrome-controlled anthocyanin biosynthesis in *Brassica oleracea*. *Phytochemistry*, **14**: 731-733.

Beggs, C.J., Kuhn, K., Böcker, R., Wellmann, E., 1987. Phytochrome-induced flavonoid biosynthesis in mustard (*Sinapis alba* L.) cotyledons. Enzymic control and differential regulation of anthocyanin and quercetin formation. *Planta*, **172**: 121-126.

Beggs, C.J., Wellmann, E., Grisebach, H., 1986. Photocontrol of flavonoid biosynthesis. In: Kendrick, R.E., Kronenberg, G.H.M. (Eds.). *Photomorphogenesis in Plants*. Martinus Nijhohh/Dr. W. Junk Publishers, Dordrecht. pp. 467-499.

Belitz, H. -D., Grosch, W., 1987. *Food Chemistry*. Springer-Verlag, Berlin.

Black, W., 1933. Studies on the inheritance of tuber colour in potatoes. *Journal of Genetics*, **27**: 319-339.

Bobbio, P.A., Bobbio, F.O., de Souza, S.C., 1987. *Solanum americanum* as a source of anthocyanins for use in foods. *Acta Alimentaria*, **16(2)**: 155-158.

Boland, M.J., Wong, E., 1976. Purification and kinetic properties of chalcone-flavonone isomerase from soya bean. *European Journal of Biochemistry*, **50**: 383-389.

Booth, A., 1963. The role of growth substances in the development of stolons. In: Ivins, J.D., Milthorpe, F.L. (Eds). *The Growth of the Potato*. Butterworths, London. pp. 99-113.

Bowen, L.H., 1994. *The Study of the Pigments in the Genus Camellia*. MSc Thesis, Canterbury University, NZ.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**: 248-254.

Braun, J., Tevini, M., 1993. Regulation of UV-protective pigment synthesis in the epidermal layer of rye seedlings (*Secale cereale* L. cv. Kustro). *Photochemistry and Photobiology*, **57(2)**: 318-323.

Britsch, L., Grisebach, H., 1986. Purification and characterization of (2*S*)-flavanone 3-hydroxylase from *Petunia hybrida*. *European Journal of Biochemistry*, **156**: 569-577.

Brödenfeldt, R., Mohr, H., 1988. Time courses for phytochrome-induced enzyme levels in phenylpropanoid metabolism (phenylalanine ammonia-lyase, naringenin-chalcone synthase) compared with time couses for phytochrome-mediated end-product accumulation (anthocyanin, quercetin). *Planta*, **176**: 383-390.

Brouillard, R., 1982. Chemical structure of anthocyanins. In: Markakis, P. (Ed.). *Anthocyanins as Food Colors*. Academic Press, New York. pp. 1-40.

Brouillard, R., 1983. The *in vivo* expression of anthocyanin colour in plants. *Phytochemistry*, **22(6)**: 1311-1323.

Brouillard, R., Mazza, G., Saad, Z., Albrecht-Gary, A.M., Cheminat, A., 1989. The copigmentation reaction of anthocyanins: a microprobe for the structural study of aqueous solutions. *Journal of the American Chemistry Society*, **111**: 2604-2610.

Brown, C.R., 1990. Modern evolution of the cultivated potato gene pool. In: Vayda, M.E., Park, W.D. (Eds.). *The Molecular and Cellular Biology of the Potato*. C.A.B. International, Wallingford, UK. pp. 1-11.

Brown, C.R., Edwards, C.G., Yang, C.-P., Dean, B.B., 1993. Orange flesh trait in potato: inheritance and carotenoid content. *Journal of the American Society for Horticultural Science*, **118(1)**: 145-150.

Brown, E., Riley, W., 1976. Greening of potato tubers: varietal response to controlled exposure to light. *Journal of the National Institute of Agricultural Botany*, **14(1)**: 70-76.

Büche, T., Sandermann, H.Jr., 1973. Lipid dependence of plant microsomal cinnamic acid 4-hydroxylase. *Archives of Biochemistry and Biophysics*, **158**: 445-447.

Burton, W.G., 1978. The physics and physiology of storage. In: Harris, P.M. (Ed.). *The Potato Crop. The Scientific Basis for Improvement*. Chapman and Hall, London. pp. 545-606.

Burton, W. G., 1989. *The Potato*, 3rd edition. Longman Scientific and Technical, Harlow, Essex, UK.

Cash, J.N., Sistrunk, W.A., Stutte, C.A., 1976. Characteristics of Concord grape polyphenoloxidase involved in juice colour loss. *Journal of Food Science*, **41**: 1398-1402.

Camm, E.L., Towers, G.H.N., 1977. Phenylalanine ammonia-lyase. In: Reinhold, L., Harborne, J.B., Swain, T. (Eds.). *Progress in Phytochemistry, Volume 4*. Pergamon Press, New York. pp. 169-188.

Castelluccio, C., Paganga, G., Melikian, N., Bolwell, G.P., Pridham, J., Sampson, J., Rice-Evans, C., 1995. Antioxidant potential of intermediates in phenylpropanoid metabolism in higher plants. *FEBS Letters*, **368**: 188-192.

Chandra, A., Nair, M.G., Iezzoni, A.F., 1993. Isolation and stabilization of anthocyanins from tart cherries (*Prunus cerasus* L.). *Journal of Agricultual and Food Chemistry*, **41(7)**: 1062-1065.

Chappell, J., Hahlbrock, K., 1984. Transcription of plant defense genes in response to UV light or fungal elicitor. *Nature*, **311**: 76-78.

Chen, L -J., Hrazdina, G., 1982. Structural transformation reactions of anthocyanins. *Experientia*, **38**: 1030-1032.

Cheng, G.W., Breen, P.J., 1991. Activity of phenylalanine ammonia-lyase (PAL) and concentrations of anthocyanins and phenolics in developing strawberry fruit. *Journal of the American Society for Horticultural Science*, **116(5)**: 865-869.

Chmielewska, Z., 1936. Sur les colorants des pommes de terre violette "Negresse". *Bulletin. Society Chimique*, **5(3)**: 1575-1588.

Coles, G.D., Lammerink, J.P., Wallace, A.R., 1993. Estimating potato crisp colour variability using image analysis and a quick visual method. *Potato Research*, **36**: 127-134.

Conner, A.J., Thomas, M.B., 1981. Re-establishing plantlets from tissue culture: a review. *International Plant Propagators' Society, Combined Proceedings for 1981*, **31**: 342-357.

Conner, A.J., Williams, M.K., Gardner, R.C., Deroles, S.C., Shaw, M.L., Lancaster, J.E., 1991. *Agrobacterium*-mediated transformation of New Zealand potato cultivars. *New Zealand Journal of Crop and Horticultural Science*, **19**: 1-8.

Coultate, T.P., 1989. *Food: The Chemistry of its Components*, 2nd edition. Royal Society of Chemistry, London.

Creasy, L.L., 1968. The significance of carbohydrate metabolism in flavonoid synthesis in strawberry leaf disks. *Phytochemistry*, 7: 1743-1749.

Creasy, L.L., 1976. Phenylalanine ammonia-lyase inactivating system in sunflower leaves. *Phytochemistry*, **15**: 673-675.

Creasy, L.L., Zucker, M., 1974. Phenylalanine ammonia-lyase and phenolic metabolism. In: Runeckles, V.C., Conn, E.E. (Eds.). *Recent Advances in Phytochemistry, Volume 8. Metabolism and Regulation of Secondary Plant Products.* Academic Press, New York. pp.1-19.

Cutter, E.G., 1978. Structure and development of the potato plant. In: Harris, P.M. (Ed.). *The Potato Crop. The Scientific Basis for Improvement.* Chapman and Hall, London. pp. 70-152.

Cvikrová, M., Sukhova, L.S., Eder, J., Korableva, N.P., 1994. Possible involvement of abscisic acid, ethylene and phenolic acids in potato tuber dormancy. *Plant Physiology and Biochemistry*, **32(5)**: 685-691.

Daravingas, G., Cain, R.F., 1968. Thermal degradation of black raspberry anthocyanin pigments in model systems. *Journal of Food Science*, **33**: 138-142.

Davies, B.H., 1976. Carotenoids. In: Goodwin, T.W. (Ed.). *Chemistry and Biochemistry of Plant Pigments*, Volume 2, 2nd Edition. Academic Press, London. pp. 38-165.

de Jong, H., 1987. Inheritance of pigmented flesh in cultivated diploid potatoes. *American Potato Journal*, **64**: 337-343.

de Jong, H., 1991. Inheritance of anthocyanin pigmentation in the cultivated potato: a critical review. *American Potato Journal*, **68(9)**: 585-593.

de Jong, H., Rowe, P.R., 1972. Genetic markers in inbred clones of cultivated diploid potatoes. *Potato Research*, **15**: 200-208.

de Sotillo, D.R., Hadley, M., Holm, E.T., 1994a. Phenolics in aqueous potato peel extract: extraction, identification and degradation. *Journal of Food Science*, **59(2)**: 649-651.

de Sotillo, D.R., Hadley, M., Holm, E.T., 1994b. Potato peel waste: stability and antioxidant activity of a freeze-dried extract. *Journal of Food Science*, **59(5)**: 1031-1033.

Debicki-Pospisil, J., Lovric, T., Trinajstic, N., Sabljic, A., 1983. Anthocyanin degradation in the presence of furfural and 5-hydroxymethylfurfural. *Journal of Food Sicence*, **49**: 411-416.

Dicosmo, F., Towers, G.H.N., 1984. Stress and secondary metabolism in cultured plant cells. *Recent Advances in Phytochemistry*, **18**: 97-175.

Diesperger, H., Müller, C.R., Sandermann, H.Jr., 1974. Rapid isolation of a plant microsomal fraction by Mg^{2+} - precipitation. *FEBS Letters*, **43(2)**: 155-158.

Dixon, R.A., Dey, P.M., Whitehead, I.M., 1982. Purification and properties of chalone isomerase from cell suspension cultures of *Phaseolus vulgaris*. *Biochimica et Biophysica Acta*, **715**: 25-33.

Dizengremel, D., 1985. Potato respiration: electron transport pathways. In: Li, P.H. (Ed.). *Potato Physiology*. Academic Press, Orlando, Florida. pp. 59-121.

Dodds, J.H., 1988. Tissue culture technology: practical application of sophisicated methods. *American Potato Journal*, **65**: 167-180.

Dodds, K.S., Long, D.H., 1955. The inheritance of colour in diploid potatoes. I. Types of anthocyanidins and their genetic loci. *Journal of Genetics*, **53**: 136-149.

Dodds, K.S., Long, D.H., 1956. The inheritance of colour in diploid potatoes. II. A three-factor linkage group. *Journal of Genetics*, **54**: 27-41.

Donnelly, D.J., Vidaver, W.E., Colbow, K., 1984. Fixation of $^{14}CO_2$ in tissue cultured red raspberry prior to and after transfer to soil. *Plant Cell Tissue and Organ Culture*, **3**: 313-317.

Duell-Pfaff, N., Wellmann, E., 1982. Involvement of phytochrome and a blue light photoreceptor in UV-B induced flavonoid synthesis in parsley (*Petroselinum hortense* Hoffm.) cell suspension cultures. *Planta*, **156**: 213-217.

Ebel, J., Hahlbrock, K., 1982. Biosynthesis. In: Harborne, J.B., Mabry, T.J. (Eds.). *The Flavonoids - Advances in Research.* Chapman and Hall, London. pp. 641-679.

Eddy, B.P., Mapson, L.W., 1951. Some factors affecting anthocyanin synthesis in cress seedlings. *Biochemical Journal*, **49**: 694-699.

Estrada, R., Tovar, P., Dodds, J.H., 1986. Induction of *in vitro* tubers in a broad range of potato genotypes. *Plant Cell, Tissue and Organ Culture*, 7: 3-10.

Faust, M., 1965. Physiology of anthocyanin development in McIntosh apple. I. Participation of pentose phosphate pathway in anthocyanin development. *Proceedings of the American Society for Horticultural Science*, **87**: 1-9.

Foy, C.D., Lee, E.H., Rowland, R., Devine, T.E., Buzzell, R.I., 1995. Ozone tolerance related to flavonol glycoside genes in soybean. *Journal of Plant Nutrition*, **18(4)**: 637-647.

Francis, F.J., 1989. Food colourants: anthocyanins. *Critical Reviews in Food Science and Nutrition*, **28**: 273-314.

Francis, F.J., 1993. Polyphenols as natural food colorants. In: Scalbert, A. (Ed.). *Polyphenolic Phenomena.* INRA Editions, Paris. pp. 209-220.

Friend, J., Reynolds, S.B., Aveyard, M.A., 1971. Phenolic metabolism in potato tuber tissue after infection with *Phytophthora infestans*. *Biochemical Journal*, **124**: 29P.

Furuya, M., Thimann, K.V., 1964. The biogenesis of anthocyanins. XI. Effects of gibberellic acid in two species of *Spirodela*. *Archives of Biochemistry and Biophysics*, **108**: 109-116.

Gage, T.B., Douglass, C.D., Wender, S.H., 1951. Identification of flavonoid compounds by filter paper chromatography. *Analytical Chemistry*, **23(11)**: 1582-1585.

Gao, L., Mazza, G., 1994. Quantitation and distribution of simple and acylated anthocyanins and other phenolics in blueberries. *Journal of Food Science*, **59(5)**: 1057-1059.

Gardiner, S., Schröder, J., Matern, U., Hammer, D., Hahlbrock, K., 1980. mRNA-dependent regulation of UDP-apiose synthase activity in irradiated plant cells. *Journal of Biological Chemistry*, **255**: 10752-10757.

Geissman, T.A., 1956. The flavonoid constituents of normal and virus infected peach and cherry leaves. *Archives of Biochemistry and Biophysics*, **60**: 21-26.

Geissman, T.A., 1962. The occurrance of flavonoid compounds in nature. In: Geissman, T.A. (Ed.). *The Chemistry of Flavonoid Compounds*. Pergamon Press, Oxford. pp. 1-5.

Gerats, A.G.M., de Vlaming, P., Dooderman, M., Al, B., Schram, A.W., 1982. Genetic control of the conversion of dihydroflavonols into flavonols and anthocyanins in flowers of *Petunia hybrida*. *Planta*, **155**: 364-368.

Goto, T., Hoshino, T., Ohba, M., 1976. Stabilization effect of neutral salts on anthocyanins, flavylium salts, anhydro bases and genuine anthocyanins. *Agricultural and Biological Chemistry*, **40**: 1593-1596.

Grayer, R.J., 1989. Flavonoids. In: Harborne, J.B. (Ed.). *Methods in Biochemistry, Volume 1. Plant Phenolics*. Academic Press, London. pp. 283-323.

Griffiths, D.W., Bain, H., Dale, M.F.B., 1995. Photo-induced changes in the total chlorogenic acid content of potato (*Solanum tuberosum*) tubers. *Journal of the Science of Food and Agriculture*, **68**: 105-110.

Grisebach, H., 1982. Biosynthesis of anthocyanins. In: Markakis, P. (Ed.). *Anthocyanins as Food Colours*. Academic Press, New York. pp. 69-92.

Gross, G.G., 1981. Phenolic acids. In: Stumpf, P.K., Conn, E.E. (Eds.). *Biochemistry of Plants: A Comprehensive Treatise, Volume 7*. Academic Press, London. pp. 301-316.

Gross, J., 1987. *Pigments in Fruits*. Academic Press, London.

Gutterson, N.C., 1993. Molecular breeding for color, flavor and fragrance. *Scientia Horticulturae,* **55**: 141-160.

Hahlbrock, K., 1981. Flavonoids. In: Conn, E.E (Ed.). *The Biochemistry of Plants, Volume 7. Secondary Plant Products.* Academic Press, New York. pp. 425-456.

Hahlbrock, K., Grisebach, H., 1975. Biosynthesis of flavonoids. In: Harborne, J.B., Mabry, T.J., Mabry, H. (Eds.). *The Flavonoids.* Academic Press, New York. pp. 866-895.

Hahlbrock, K., Scheel, P., 1989. Physiology and molecular biology of phenylpropanoid metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology,* **40**: 347-369.

Hahlbrock, K., Ebel, J., Ortmann, R., Sutter, A., Wellmann, E., Grisebach, H., 1971a. Regulation of enzyme activities related to the biosynthesis of flavone glycosides in cell suspension cultures of parsley (*Petroselinum hortense*). *Biochimica et Biophysica Acta,* **244**: 7-15.

Hahlbrock, K., Sutter, A., Wellmann, E., Ortmann, R., Grisebach, H., 1971b. Relationship between organ development and activity of enzymes involved in flavone glycoside biosynthesis in young parsley plants. *Phytochemistry,* **10**: 109-116.

Hahlbrock, K., Knobloch, K.-H., Kreuzaler, F., Potts, J.R.M., Wellmann, E., 1976. Co-ordinated induction and subsequent activity changes of two groups of metabolically interrelated enzymes. Light-induced synthesis of flavonoid glycosides in cell suspension cultures of *Petroselinum hortense*. *European Journal of Biochemistry,* **61**: 199-206.

Hanson, K.R., Havir, E.A., 1979. An introduction to the enzymology of phenylpropanoid biosynthesis. In: Swain, T., Harborne, J.B., van Sumere, C.F. (Eds.). *Recent Advances in Phytochemistry, Volume 12. Biochemistry of Plant Phenolics.* Plenum Press, New York. pp. 91-137.

Hanson, K.R., Havir, E.A., 1981. Phenylalanine ammonia-lyase. In: Conn, E.E. (Ed.). *The Biochemistry of Plants, Volume 7. Secondary Plant Products.* Academic Press, New York. pp. 577-625.

Harborne, J.B., 1959. The chromatography of the flavonoid pigments. *Journal of Chromatography,* **2**: 581-604.

Harborne, J.B., 1960a. Plant polyphenols. I. Anthocyanin production in the cultivated potato. *Biochemical Journal,* **74**: 262-269.

Harborne, J.B., 1960b. Plant polyphenols. II. The coumarins of *Solanum pinnatisectum*. *Biochemical Journal*, **74**: 270-273.

Harborne, J.B., 1962. Chemicogenetical studies of flavonoid pigments. In: Geissman, T.A. (Ed.). *The Chemistry of Flavonoid Compounds*. Pergamon Press, Oxford. pp. 593-617.

Harborne, J.B., 1964. Plant polyphenols - XI. The structure of acylated anthocyanins. *Phytochemistry*, **3**: 151-160.

Harborne, J.B., 1967. *Comparative Biochemistry of the Flavonoids*. Academic Press, London.

Harborne, J.B., 1972. Evolution and function of flavonoids in plants. In: Runeckles, V.C., Watkin, J.E. (Eds.). *Recent Advances in Phytochemistry, Volume 4*. Appleton-Century-Crofts, New York. pp. 107-141.

Harborne, J.B., 1976. Functions of flavonoids in plants. In: Goodwin, T.W. (Ed.). *Chemistry and Biochemistry of Plant Pigments, Volume I*. Academic Press, London. pp. 736-778.

Harborne, J.B., 1988. The flavonoids: recent advances. In: Goodwin, T.W. (Ed.). *Plant Pigments*. Academic Press, London. pp. 299-343.

Harborne, J.B., 1989. General procedure and measurement of total phenolics. In: Harborne, J.B. (Ed.). *Methods in Plant Biochemistry, Volume 1. Plant Phenolics*. Academic Press, London. pp. 1-28.

Harborne, J.B., Sherratt, H.S.A., 1957. Variations in the glycosidic pattern of anthoycanins, Part II. *Experientia*, **13**: 486-487.

Harvey, B.M.R., Crothers, S.H., Evans, H.E., Selby, C., 1991. The use of growth retardants to improve microtuber formation by potato (*Solanum tuberosum*). *Plant Cell, Tissue and Organ Culture*, **27**: 59-64.

Havir, E.A., Hanson, K.R., 1968. L-Phenylalanine ammonia-lyase. I. Purification and molecular size of the enzyme from potato tubers. *Biochemistry*, **7(5)**: 1896-1903.

Havir, E.A., Hanson, K.R., 1973. L-Phenylalanine ammonia-lyase (maize and potato). Evidence that the enzyme is composed of four subunits. *Biochemistry*, **12(8)**: 1583-1591.

Hawkes, J.G., 1989. Nomenclatural and taxonomic notes on the infrageneric taxa of the tuber-bearing *Solanums* (Solanaceae). *Taxon*, **39(3)**: 489-492.

Hawkes, J.G., 1990. *The Potato. Evolution, Biodiversity and Genetic Resources.* Belhaven Press, London.

Hebash, K.A., Fadel, H.H.M., 1994. Changes in oil and potato chips during frying. *Die Nahrung*, **3**: 278-282.

Heinsbroek, R., van Brederode, J., van Nigtevecht, G., Kamsteeg, J., 1979. Biosynthesis and genetic control of isovitexin 2'-*O*-arabinoside in petals of *Silene dioica*. *Phytochemistry*, **18**: 935-937.

Helariutta, Y., Elomaa, P., Kotilainen, M., Grisebach, R.J., Schröder, J., Teeri, T.H., 1995. Chalcone synthase-like genes active during corolla development are differentially expressed and encode enzymes with different catalytic properties in *Gerbera hybrida* (Asteraceae). *Plant Molecular Biology*, **28**: 47-60.

Heller, W., Forkmann, G., 1988. Biosynthesis. In: Harborne, J.B. (Ed.). *The Flavonoids.* Chapman and Hall Ltd, London. pp. 399-425.

Heller, W., Hahlbrock, K., 1980. Highly purified "flavonone synthase" from parsley catalyzes the formation of naringenin chalcone. *Archives of Biochemistry and Biophysics*, **20**: 617-619.

Heller, W., Britsch, L., Forkmann, G., Grisebach, H., 1985a. Leucoanthocyanidins as intermediates in anthocyanin biosynthesis in flowers of *Matthiola incana* R. Br. *Planta*, **163**: 191-196.

Heller, W., Forkmann, G., Britsch, L., Grisebach, H., 1985b. Enzymatic reduction of (+)-dihydroflavonols to flavan-3,4-*cis*-diols with flower extracts from *Matthiola incana* and its role in anthocyanin biosynthesis. *Planta*, **165**: 284-287.

Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.B., Kromhout, D., 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *The Lancet*, **342**: 1007-1011.

Hertog, M.G.L., Kromhout, D., Aravanis, C., Blackburn, H., Buzina, R., Fidanza, F., Giampaoli, S., Jansen, A., Menotti, A., Nedeljkovic, S., Pettarinen, M., Simic, B.S., Toshima, H., Freskens, E.J.M., Hollman, P.C.H., Katan, M.B., 1995. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Archives of Internal Medicine*, **155(4)**: 381-386.

Heyer, A., Gatz, C., 1992. Isolation and characterization of a cDNA-clone coding for potato type A phytochrome. *Plant Molecular Biology*, **18**: 535-544.

Hinderer, W., Petersen, M., Seitz, H.U., 1984. Inhibition of flavonoid biosynthesis by gibberellic acid in cell suspension cultures of *Daucus carota* L. *Planta,* **160**: 544-549.

Houwing, A., Suk, R., Ros, B., 1986. Generation of lightsprouts suitable for potato variety identification by means of artificial light. *Acta Horticulturae*, **182**: 359-363.

Howard, H.W., 1960. Potato cytology and genetics, 1952-1959. *Bibliographia Genetica*, **19**: 87-216.

Howard, H.W., 1962. Experiments with potatoes on the effect of the pigment-restricting gene - M. *Heredity*, **17(2)**: 145-156.

Howard, H.W., 1964. Further effects of the pigment-restricting gene, M, in potatoes: hidden spotted and spectacled. *Heredity*, **19(3)**: 349-356.

Howard, H.W., 1969. *Genetics of the potato Solanum tuberosum*. Logos Press, London.

Howard, H.W., Kukimura, H., Whitmore, E.J., 1970. The anthocyanin pigments of the tubers and sprouts of *Tuberosum* potatoes. *Potato Research*, **13**: 142-145.

Hrazdina, G., 1982. Anthocyanins. In: Harborne, J.B., Mabry, T.J. (Eds.). *The Flavonoids, Advances in Research*. Chapman and Hall, New York. pp. 135-188.

Hrazdina, G., 1992. Compartmentation in aromatic metabolism. In: Stafford, H.A., Ibrahim, R.K. (Eds.). *Recent Advances in Phytochemistry, Volume 26. Phenolic Metabolism in Plants.* Plenum Press, New York. pp. 1-23.

Hrazdina, G., Wagner, G.J., 1985a. Compartmentation of plant phenolic compounds: sites of synthesis and accumulation. *Annual Proceedings of the Phytochemical Society of Europe*, **25**: 120-123.

Hrazdina, G., Wagner, G., 1985b. Metabolic pathways as enzyme complexes: evidence for the synthesis of phenylpropanoids and flavonoids on membrane associated enzyme complexes. *Archives of Biochemistry and Biophysics,* **237(1)**: 88-100.

Husain, S.R., Cillard, J., Cillard, P., 1987. Hydroxy radical scavenging activity of flavonoids. *Phytochemistry*, **26**: 2489-2492.

Hussey, G., Stacey, N.J., 1984. Factors affecting the formation of *in vitro* tubers of potato (*Solanum tuberosum* L.). *Annals of Botany*, **53**: 565-578.

Irwin, P.L., Pfeffer, P.E., Doner, L.W., Sapers, G.M., Brewster, J.D., Nagahashi, G., Hicks, KB., 1994. Binding geometry, stoichiometry, and thermodynamics of cyclomalto-oligosaccharide (cyclodextrin) inclusion complex formation with chlorogenic acid, the major substrate of apple polyphenol oxidase. *Carbohydrate Research*, **256**: 13-27.

Isherwood, F.A., 1976. Mechanism of starch-sugar interconversion in *Solanum tuberosum*. *Phytochemistry*, **15**: 33-41

Ishizuka, M., Yamada, F., Tanaka, Y., Takeuch, Y., Imaseki, H., 1991. Sequential induction of mRNAs for phenylalanine ammonia-lyase in slices of potato tuber. *Plant and Cell Physiology*, **32(1)**: 57-64.

Jackman, R.L., Yada, R.Y., Tung, M.A., Speers, R.A., 1987. Anthocyanins as food colourants - a review. *Journal of Food Biochemistry*, **11**: 201-247.

Jensen, R.A., 1985. The shikimate/arogenate pathway: link between carbohydrate metabolism and secondary metabolism. *Physiologia Plantarum*, **66**: 164-168.

Jorgensen, R., 1994. The genetic origins of biosynthesis and light-responsive control of the chemical UV screen of land plants. In: Ellis, B.E., Kuroki, G.W., Stafford, H.A. (Eds.). *Recent Advances in Phytochemistry, Volume 28. Genetic Engineering of Plant Secondary Metabolism.* Plenum Press, New York. pp. 179-192.

Jurd, L., Asen, S., 1966. The formation of metal and "co-pigment" complexes of cyanidin-3-glucoside. *Phytochemistry*, **5**: 1263-1271.

Kennedy, J.F., White, C.A., 1979. Polysaccharides. In: Haslam, E. (Ed.). *The Synthesis and Reactions of Organic Compounds, Volume 5. Biological Compounds.* Pergamon Press, Oxford. pp. 755-815.

Kessel, R., Rowe, P.R., 1974. Inheritance of two quantitative traits and a proposed genetic map for their linkage group in diploid potatoes. *Potato Research*, **17**: 283-295.

Khanbari, O.S., Thompson, A.K., 1993. Effects of amino acids and glucose on the fry colour of potato crisps. *Potato Research*, **36(4)**: 359-364.

Kling, G.J., Meyer, M.M.Jr., 1983. Effects of phenolic compounds and indolacetic acid in adventitious root initiation in cuttings of *Phaseolus aurus*, *Acer saccharinum*, and *Acer griseum*. *HortScience*, **18(3)**: 352-354.

Knogge, W., Weissenböck, G., 1986. Tissue-distribution of secondary phenolic biosynthesis in developing primary leaves of *Avena sativa* L. *Planta*, **167**: 196-205.

Koukol, J., Conn, E.E., 1961. The metabolism of aromatic compounds in higher plants IV. Purification and properties of the phenylalanine deaminase of *Hordeum vulgare*. *Journal of Biological Chemistry*, **236(10)**: 2692-2698.

Krauss, A., 1985. Interaction of nitrogen nutrition, phytohormones, and tuberization. In: Li, P.H. (Ed.). *Potato Physiology*. Academic Press, Orlando, Florida. pp. 209-230.

Kreuzaler, F., Ragg, H., Fautz, E., Kuhn, D.N., Hahlbrock, K., 1983. UV-induction of chalcone synthase mRNA in cell suspension cultures of *Petroselinum hortense*. *Proceedings of the National Academy of Sciences - USA*, **80**: 2591-2593.

Kuc, J., 1957. A biochemical study of the resistance of potato tuber tissue to attack by various fungi. *Phytopathology*, **47**: 676-680.

Kuc, J., Henze, R.E., Ullstrup, A.J., Quackenbush, F.W., 1956. Chlorogenic and caffeic acids as fungistatic agents produced by potatoes in response to inoculation with *Helminthosporium carbonium*. *Journal of the American Chemical Society*, **78(5)**: 3123-3125.

Kuhn, D.N., Chappell, J., Boudet, A., Hahlbrock, K., 1984. Induction of phenylalanine ammonia-lysae and 4-coumarate:CoA ligase mRNAs in cultured plant cells by UV light and a fungal elicitor. *Proceedings of the National Academy of Sciences - USA*, **81**: 1102-1106.

Kumar, A., Pundhir, V.S., Gupta, K.C., 1991. The role of phenols in potato tuber resistance against soft rot by *Erwinia carotovora* ssp. *carotovora*. *Potato Research*, **34**: 9-16.

Lamb, C.J., 1979. Regulation of enzyme levels in phenlypropanoid biosynthesis: characterization of the moduation by light and pathway intermediates. *Archives of Biochemistry and Biophysics*, **192(1)**: 311-317.

Lamb, C.J., Merritt, T.K., 1979. Density labelling studies of the photocontrol of L-phenylalanine ammonia lyase in disks of potato (*Solanum tuberosum*) tuber parenchyme. *Biochimica et Biophysica Acta*, **588**: 1-11.

Lamb, C.J., Rubery, P.H., 1975. A spectrophotometric assay for *trans*-cinnamic acid 4-hydroxylase activity. *Analytical Biochemistry*, **68**: 554-561.

Lamb, C.J., Rubery, P.H., 1976. Photocontrol of chlorogenic acid biosynthesis in potato tuber discs. *Phytochemistry*, **15**: 665-668.

Lancaster, J.E., Grant, J.E., Lister, C.E., Taylor, M.C., 1994. Skin colour in apples - influence of copigmentation and plastid pigments on shade and darkness of red colour in five genotypes. *Journal of the American Society for Horticultural Science*, **119(1)**: 63-69.

Larson, R.A., 1995. Plant defenses against oxidative stress. *Archives of Insect Biochemistry and Physiology*, **29**: 175-186.

Lee, S.F., Le Tourneau, D., 1958. Chlorogenic acid content and *Verticullium* wilt resistance of potatoes. *Phytopathology*, **48**: 268-274.

Leibrand, J.T., Smiles, R.E., Freeman, T.M., 1985. Functions and applications for polydextrose in foods. *Food Technology in Australia*, **37(4)**: 166-167.

Lewis, C.E., Walker, J.R.L., Lancaster, J.E., 1995. Effect of polysaccharides on the colour of anthocyanins. *Food Chemistry*, **54**: 315-319.

Lewis, C.E., Walker, J.R.L., Lancaster, J.E., 1996. Effect of cooking treatments on pigment loss from coloured potato tubers. *The Food Technologist.* Submitted.

Liang, X., Dron, M., Cramer, C.L., Dixon, R.A., Lamb, C.J., 1989. Differential regulation of phenylalanine ammonia-lyase genes during plant development and environmental cues. *Journal of Biological Chemistry*, **264(24)**: 14486-14492.

Lister, C.E., 1994. *Biochemistry of Fruit Colour in Apples (Malus pumila Mill.).* PhD Thesis, University of Canterbury, NZ.

Lizarraga, R., Huaman, Z., Dodds, J.H., 1989. *In vitro* conservation of potato germplasm at the International Potato Centre. *American Potato Journal*, **66**: 253-269.

Lommen, W.J.M., Struik, P.C., 1994. Field performance of potato minitubers with different fresh weights and conventional seed tubers: crop establishment and yield formation. *Potato Research*, **37**: 301-313.

Lulai, E.C., Orr, P.H., 1994. Techniques for detecting and measuring developmental and maturational changes in tuber native periderm. *American Potato Journal*, **71**: 489-505.

Lyon, G.D., Barker, H., 1984. The measurement of chlorogenic acid in potato leaf extracts by high-pressure liquid chromatography. *Potato Research*, **27**: 291-295.

Mabry, T.J., Markham, K.R., Thomas, M.B., 1970. *The Systematic Identification of Flavonoids.* Springer-Verlag, New York.

Mahmood, N., Pizza, C., Aquino, R., de Tommasi, N., Piacente, S., Colman, S., Burke, A., Hay, A.J., 1993. Inhibition of HIV infection by flavonoids. *Antiviral Research*, **22**: 189-199.

Malmberg, A.G., Theander, O., 1985. Determination of chlorogenic acid in potato tubers. *Journal of Agricultural and Food Chemistry*, **53**: 549-551.

Malterud, K.E., Bremnes, T.E., Faegri, A., Moe, T., Sandanger-Dugstad, E.K., Anthonsen, T., Henriksen, L.M., 1985. Flavonoids from the wood of *Salix caprea* as inhibitors of wood-destroying fungi. *Journal of Natural Products*, **48(4)**: 559-563.

Mancinelli, A.L., 1985. Light-dependent anthocyanin synthesis: a model system for the study of plant photomorphogenesis. *Botanical Review*, **51(1)**: 107-157.

Mancinelli, A.L., Rossi, F., Moroni, A., 1991. Cryptochrome, phytochrome, and anthocyanin production. *Plant Physiology*, **96**: 1079-1085.

Margna, U., 1977. Control at the level of substrate supply - an alternative in the regulation of phenylpropanoid accumulation in plant cells. *Phytochemistry*, **16**: 419-426.

Markakis, P., 1974. Anthocyanins and their stability in foods. In: *Critical Reviews in Food Science and Nutrition*. CRC Press, Boca Raton, Florida. pp. 437-456.

Markakis, P., Livingston, G.E., Fellers, C.R., 1957. Quantitative aspects of strawberry pigment degradation. *Food Research*, **22**: 117-130.

Markham, K.R., 1982. *Techniques of Flavonoid Identification.* Academic Press, London.

Mazza, G., Brouillard, R., 1987. Recent developments in the stabilization of anthocyanins in food products. *Food Chemistry*, **25**: 207-225.

Mazza, G., Brouillard, R., 1990. The mechanism of co-pigmentation of anthocyanins in aqueous solutions. *Phytochemistry*, **29(4)**: 1097-1102.

Mazza, G., Miniati, E., 1993. *Anthocyanins in Fruits, Vegetables and Grains*, CRC Press, London.

McClure, J.W., 1975. Physiology and functions of flavonoids. In: Harborne, J.B., Mabry, T.J., Mabry, H. (Eds.). *The Flavonoids.* Chapman and Hall, London. pp. 970-1055.

McClure, J.W., 1986. Physiology of flavonoids in plants. In: Cody, V., Middleton, E.Jr., Harborne, J.B. (Eds.). *Progress in Clinical and Biological Research, Volume 213. Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological ,and Structure-Activity Relationships.* Alan R. Liss, Inc, New York. pp. 77-85.

McRae, D.C., Glasbey, C.A., Melrose, H., Fleming, J., 1986. Size grading methods and their relationship to the dimensions, mass and volume characteristics if potato cultivars. *Potato Research*, **29**: 477-486.

Melis, R.J.M., van Staden, J., 1984. Tuberization and hormones. *Zeitschrift für Pflanzenphysiologie*, **113(3)**: 271-283.

Meredith, P., 1989. Describing size and shape of potato tubers by numbers. *Lebensmittel-Wissenschaft Technologie*, **22**: 368-375.

Meredith, P., 1995. A shapely response to the chippers. *Potato Review*, **5(2)**: 36-38.

Meschter, E.E., 1953. Fruit colour loss. Effects of carbohydrates and other factors on strawberry products. *Journal of Agricultural and Food Chemistry*, **1**: 574-579.

Middleton, E.Jr., Kandaswami, C., 1994. Potential health-promoting properties of *Citrus* flavonoids. *Food Technology*, **48(11)**: 115-119.

Middleton, E.M., Teramura, A.H., 1993. The role of flavonol glycosides and carotenoids in protecting soybean from ultraviolet-B damage. *Plant Physiology*, **103**: 741-752.

Mingo-Castel, A.M., Smith, O.E., Kumamoto, J., 1976. Studies on the carbon dioxide promotion and ethylene inhibition of tuberization in potato explants cultured *in vitro*. *Plant Physiology*, **57**: 480-485.

Mol, J.N.M., Robbins, M.P., Dixon, R.A., Veltkamp, E., 1985. Spontaneous and enzymic rearrangement of naringenin chalcone to flavanone. *Phytochemistry*, **24(10)**: 2267-2269.

Moorby, J., 1978. The physiology of growth and tuber yield. In: Harris, P.M. (Ed.). *The Potato Crop. The Scientific Basis for Improvement.* Chapman and Hall, London. pp. 153-194.

Morrison, W.R., Karkalas, J., 1990. Starch. In: Dey, P.M. (Ed.), *Methods in Plant Biochemistry 2, Carbohydrates.* Academic Press, London. pp. 323-352.

Moustafa, E., Wong, E., 1967. Purification and properties of chalcone-flavanone isomerase from soya bean seed. *Phytochemistry,* **6**: 625-632.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, **15**: 473-497.

Nagai, N., Kitauchi, F., Okamoto, K., Kanda, T., Shimosaka, M., Okazaki, M., 1994. A transient increase of phenylalanine ammonia-lyase transcript in kinetin-treated tobacco callus. *Bioscience, Biotechnology and Biochemistry,* **58(3)**: 558-559.

Nakagami, T., Nanaumi-Tamuia, N., Toyomura, K., Nakamura, T., Shigehisa, T., 1995. Dietary flavonoids as potential natural biological response modifiers affecting the autoimmune system. *Journal of Food Science*, **60(4)**: 653-656.

Neumann, K., -H., Barz, W., Reinhard, E. (Eds.), 1985. *Primary and Secondary Metabolism of Plant Cell Cultures.* Springer-Verlag, Berlin.

Olsson, K., Jonasson, T., 1995. Genotypic differences in susceptibility to wireworm attack in potato: mechanisms and implications for plant breeding. *Plant Breeding*, **114**: 66-69.

Onyeneho, S.N., Hettiarachchy, N.S., 1993. Antioxidant activity, fatty acids and phenolic acids compositions of potato peels. *Journal of the Science of Food and Agriculture*, **62**: 345-350.

Osawa, Y., 1982. Copigmentation of anthocyanins. In: Markakis, P. (Ed.). *Anthocyanins as Food Colors.* Academic Press, New York. pp. 41-68.

Peng, C.Y., Markakis, P., 1963. Effect of phenolase on anthocyanins. *Nature*, **199**: 597-598.

Peterson, R.L., Barker, W.G., 1979. Early tuber development from explanted stolon nodes of *Solanum tuberosum* var. Kennebec. *Botanical Gazette (Chicago)*, **140(4)**: 398-406.

Peterson, R.L., Barker, W.G., Howarth, M.J., 1985. Development and structure of tubers. In: Li, P.H. (Ed.). *Potato Physiology.* Academic Press, Orlando, Florida. pp. 123-152.

Phillips, D.A., 1992. Flavonoids: plant signals to soil microbes. In: Stafford, H.A., Ibrahim, R.K. (Eds.). *Recent Advances in Phytochemistry, Volume 26. Phenolic Metabolism in Plants.* Plenum Press, New York. pp. 201-231.

Picinelli, A., Dapena, E., Mangas, J.J., 1995. Polyphenolic pattern in apple tree leaves in relation to scab resistance. A preliminary study. *Journal of Agricultural and Food Chemistry*, **43(8)**: 2273-2278

Platt, H.W., Bollen, G.J., 1993. Use of tissue culture potato plantlets for investigation of diseases of subterranean plant parts. *Plant Disease*, **77(11)**: 1112-1113.

Porter, L.J., 1988. Flavans and proanthocyanidins. In: Harborne, J.B. (Ed.). *The Flavonoids.* Chapman and Hall, London. pp. 21-62.

Quinn, J.S., Schafer, H.W., 1994. Characterization of pectic substances from two potato cultivars with different sensitivities to prewarming. *Potato Research*, **37(1)**: 87-97.

Ramamurthy, M.S., Maiti, B., Thomas, P., Nair, P.M., 1992. High-performance liquid chromatography determination of phenolic acids in potato tubers (*Solanum tuberosum*) during wound healing. *Journal of Agricultural and Food Chemistry*, **40**: 569-572.

Ranalli, P., Bassi, F., Ruaro, G., Del Re, P., Dicandilo, M., Mandolino, G., 1994. Microtuber and minituber production and field performance compared with normal tubers. *Potato Research*, **37(4)**: 383-391.

Read, M.A., 1995. Flavonoids: naturally occurring anti-inflammatory agents. *American Journal of Pathology*, **147(2)**: 235-237.

Reddy, V.S., Goud, K.V., Sharma, R., Reddy, A.R., 1994. Ultraviolet-B-responsive anthocyanin production in a rice cultivar is associated with a specific phase of phenylalanine ammonia lyase biosynthesis. *Plant Physiology*, **105**: 1059-1066.

Reeve, R.M., Hautala, E., Weaver, M. L., 1969a. Anatomy and compositional variation within potatoes. II. Phenolics, enzymes and other minor components. *American Potato Journal*, **46**: 374 -386.

Reeve, R.M., Hautala, E., Weaver, M. L., 1969b. Anatomy and compositional variation within potatoes. I. Developmental histology of the tuber. *American Potato Journal*, **46**: 361-373.

Reeve, R.M., Hautala, E., Weaver, M. L., 1970. Anatomy and compositional variation within potatoes. III. Gross compositional gradients. *American Potato Journal*, **47**: 148-162.

Ribéreau-Gayon, P., 1972. *Plant Phenolics.* Oliver and Boyd, Edinburgh.

Robinson, D.S., 1987. *Food: Biochemistry and Nutritional Value.* Longman Scientific and Technical, Harlow, Essex, UK.

Russell, D.W., 1971. The metabolism of aromatic compounds in higher plants. X. Properties of the cinnamic acid 4-hydroxylase of pea seedlings and some aspects of its metabolic and developmental control. *Journal of Biological Chemistry*, **246(12)**: 3870-3878.

Sacher, J.A., Towers, G.H.N., Davies, D.D., 1972. Effect of light and ageing on enzymes, particularly phenylalanine ammonia lyase, in discs of storage tissue. *Phytochemistry*, **11**: 2383-2391.

Sachse, J., 1973. The anthocyanins in the potato varieties Urgenta and Desirée (*Solanum tuberosum* L.). *Zeitschrift fur Lebensmittel Untersuchung und Forschung*, **153**: 294-300.

Saenger, W., 1980. Cyclodextrin inclusion compounds in research and industry. *Angewandte Chemie. International Edition in English*, **19**: 344-362.

Salaman, R.N., 1910. The inheritance of colour and other characters of the potato. *Journal of Genetics*, 1: 7-46.

Salisbury, F.B., Ross, C.W., 1992. *Plant Physiology*, 4th edition. Wadsworth, Belmont, California.

Scanlon, M.G., Roller, R., Mazza, G., Pritchard, M.K., 1994. Computerized video image analysis to quantify color of potato chips. *American Potato Journal*, **71**: 717-733.

Schmidt, H. W., Schönherr, J., 1982. Fine structure of isolated and non-isolated potato tuber periderm. *Planta*, **154**: 76-80.

Schröder, J., 1977. Light-induced increase of messenger RNA for phenylalanine ammonia-lyase in cell suspension cultures of *Petroselinum hortense*. *Archives of Biochemistry and Biophysics*, **182**: 488-496.

Schröder, J., Heller, W., Hahlbrock, K., 1979. Flavanone synthase: a simple and rapid assay for the key enzyme of flavonoid biosynthesis. *Plant Science Letters*, **14**: 281-286.

Scott, R.K., Wilcockson, S.J., 1978. Application of physiological and agronomic principles to the development of the potato industry. In: Harris, P.M. (Ed.). *The Potato Crop. The Scientific Basis for Improvement.* Chapman and Hall, London. pp. 678-704.

Sebastiani, L., Lenzi, A., Pugliesi, C., Fambrini, M., 1994. Somaclonal variation for resistance to *Verticillium dahliae* in potato (*Solanum tuberosum* L.) plants regenerated from callus. *Euphytica*, **80(1-2)**: 5-11.

Shock, C.C., Stieber, T.D., Zalewski, J.C., Eldredge, E.P., Lewis, M.D., 1994. Potato tuber stem-end fry color determination. *American Potato Journal*, **71(2)**: 77-88.

Siddiq, M., Arnold, J.F., Sinha, N.K., Cash, J.N., 1994. Effect of polyphenol oxidase and its inhibitors on anthocyanin changes in plum juice. *Journal of Food Processing and Preservation*, **18(1)**: 75-84.

Siegel, P., Markakis, P., Bedford, C.L., 1971. Stabilization of anthocyanins in frozen tart cherries by blanching. *Journal of Food Science*, **36**: 962-963.

Simmonds, N.W., 1976. Potatoes. In: Simmonds, N.W. (Ed.). *Evolution of Crop Plants*. Longman, London. pp. 279-283.

Simmonds, N.W., Harborne, J.B., 1965. Control of malvidin synthesis in the cultivated potatoes. *Heredity*, **20**: 315-318.

Sirks, M.J., 1929. The inheritance of some anthocyane-factors in the potato. *Genetica*, **11**: 293-328.

Smith, H., 1972. The photocontrol of flavonoid biosynthesis. In: Mitrakos, K., Shropshire, W. (Eds.). *Phytochrome*. Academic Press, New York. pp. 433-481.

Smith, H.B., 1927. Chromosome counts in the varieties of *Solanum tuberosum* and allied wild species. *Genetics*, **12**: 84-92.

Sorota, Y., Takahama, U., Kimura, M., 1982. Protective effect of quercetin and rutin on photosensitized lysis of human erythrocytes in the presence of hematoporphyrin. *Biochimica et Biophysica Acta*, **799**: 313-317.

Sosulski, F., Krygier, K., Hogge, L., 1982. Free, esterified, and insoluble-bound phenolic acids. 3. Composition of phenolic acids in cereal and potato flours. *Journal of Agricultural and Food Chemistry*, **30**: 337-340.

Sowokinos, J., 1990a. Stress-induced alterations in carbohydrate metabolism. In: Vayda, M.E., Park, W.D. (Eds.). *The Molecular and Cellular Biology of the Potato*. C.A.B. International, Wallingford, UK. pp. 137-158.

Sowokinos, J., 1990b. Effect of stress and senesence on carbon partitioning in stored potatoes. *American Potato Journal*, **67**: 849-857.

Sparks, G.A., 1990. *Identification of New Zealand Potato Cultivars.* MAF Quality Management, Lincoln, NZ.

Stachel, S.E., Messens, E., van Montagu, M., Zambryski, P., 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature*, **318**: 624-629.

Stachel, S.E., Nester, E.W., Zambryski, P.C., 1986. A plant cell factor induces *Agrobacterium tumefaciens* vir gene expression. *Proceedings of the National Academy of Sciences - USA*, **84**: 7428-7432.

Stafford, H.A., 1974. The metabolism of aromatic compounds. *Annual Review of Plant Physiology*, **25**: 459-486.

Stafford, H.A., 1988. Proanthocyanidins (PAs) - and the lignin connection. *Phytochemistry*, **27**: 1-6.

Stafford, H.A., 1990. *Flavonoid Metabolism*, CRC Press, Boca Raton, Florida.

Stafford, H.A., 1993. Proanthocyanin and flavan-3-ol biosynthesis: comparison with the anthocyanidin pathway and future needs. In: Scalbert, A. (Ed.). *Polyphenolic Phenomena*. INRA Editions, Paris. pp. 73-80.

Stafford, H.A., Lester, H.H., 1982. Enzymic and nonenzymic reduction of (+)-dihydroquercetin to its 3,4-diol. *Plant Physiology*, **70**: 695-698.

Stafford, H.A., Lester, H.H., 1984. Flavan-3-ol biosynthesis. The conversion of (+)-dihydroquercetin and flavan-3,4-cis-diol (leucocyanidin) to (+)-catechin by reductases extracted from cell suspension cultures of Douglas fir. *Plant Physiology*, **76**: 184-186.

Stafford, H.A., Lester, H.H., 1985. Flavan-3-ol biosynthesis. The conversion of (+)-dihydromyricetin to its flavan-3,4-diol (leucodelphinidin) and to (+)-gallocatechin by reductases extracted from tissue cultures of *Ginkco biloba* and *Pseudotsuga menziesii*. *Plant Physiology*, **78**: 791-794.

Stallknecht, G.F., 1985. Tuber initiation in *Solanum tuberosum*: effect of phytohormones and induced changes in nucleic acid and protein metabolism. In: Li, P.H. (Ed.). *Potato Physiology*. Academic Press, Orlando, Florida. pp. 232-260.

Starr, M.S., Francis, F.J., 1973. Effect of metallic ions on colour and pigment content of cranberry juice cocktail. *Journal of Food Science*, **38**: 1043-1046.

Stewart, R.N., Norris, K.H., Asen, S., 1975. Microspectrophotometric measurement of pH and pH effect on colour of petal epidermal cells. *Phytochemistry*, **14**: 937-942.

Strack, D., Wray. V., 1989. Anthocyanins. In: Harborne, J.B. (Ed.). *Methods in Plant Biochemistry, Volume 1. Plant Phenolics.* Academic Press, London. pp. 325-356.

Strack, D., Heilemann, J., Wray, V., Dirks, H., 1989. Structures and accumulation patterns of soluble and insoluble phenolics from Norway spruce needles. *Phytochemistry*, **28**: 2071-2078.

Street, H.E., Öpik, H., 1970. *The Physiology of Flowering Plants: Their Growth and Development.* Edward and Arnold, London.

Strid, A., Chow, W.S., Anderson, J.M., 1994. UV-B damage and protection at the molecular level in plants. *Photosynthesis Research*, **39(3)**: 475-489.

Swain, T., 1962. Economic importance of flavonoid compounds: foodstuffs. In: Geissman, T.A. (Ed.). *The Chemistry of Flavonoid Compounds.* Pergamon Press, Oxford. pp. 513-552.

Swain, T., 1979. Tannins and lignins in herbivores. In: Rosenthal, G.A., Janzen, D.H. (Eds.). *The Herbivores, Their Interactions with Secondary Plant Metabolites.* Academic Press, New York. p. 659-687.

Swaminathan, M.S., Howard, H.W, 1953. The cytology and genetics of the potato (*Solanum tuberosum*) and related species. *Bibliographia Genetica*, **16**: 1-192.

Szejtli, V.J., 1978. Neue untersuchungsmethoden in der cyclodextrinchemie. *Starch/Stärke*, **30**: 427-431.

Takahama, U., 1985. Inhibition of lipoxygenase-dependent lipid peroxidation by quercetin: mechanism of antioxidative function. *Phytochemistry*, **24**: 1443-1446.

Tan, S.C., 1979. Relationships and interactions between phenylalanine ammonia-lyase, phenylalanine ammonia-lyase inactivating system, and anthocyanin in apples. *Journal of the American Society for Horticultural Science*, **104(5)**: 581-586.

Tanaka, Y., Matsushita, K., Uritani, I., 1977. Some investigations on inactivation of phenylalanine ammonia-lyase in cut injured sweet potato root tissue. *Plant and Cell Physiology.* **18**: 1209-1216.

Teitelbaum, R.C., Ruby, S.L., Marks, T.J., 1978. On the structure of starch iodine. *Journal of the American Chemical Society*, **100**: 3215-3217.

Teitelbaum, R.C., Ruby, S.L., Marks, T.J., 1980. A resonance Raman/iodine Mössbauer investigation of the starch-iodine structure. Aqueous solution and iodine vapour preparations. *Journal of the American Chemical Society*, **102**: 3322-3328.

Tevini, M., Braun, J., Fieser, G., 1991. The protective function of the epidermal layer of rye seedlings against ultraviolet-B radiation. *Photochemistry and Photobiology*, **53(3)**: 329-333.

Thimann, K.V., Edmondson, Y.H., Radner, B.S., 1951. The biogenesis of the anthocyanins. III. The role of sugars in anthocyanin formation. *Acrhives of Biochemistry and Biophysics*, **34**: 305-323.

Timberlake, C.F., 1980. Anthocyanins - occurrence, extraction and chemistry. *Food Chemistry*, **5**: 69-80.

Timberlake, C.F., Bridle, P., 1966. Spectral studies of anthocyanin and anthocyanidin equilibria in aqueous solution. *Nature*, **212**: 158-159.

Timberlake, C.F., Bridle, P., 1982. Distribution of anthoycanins in food plants. In: Markakis, P. (Ed.). *Anthocyanins as Food Colours*. Academic Press, New York. pp. 125-162.

Tissut, M., Chevallier, D., Douce, R., 1980. Effet de différents polyphénols sur les mitochondries et les chloroplastes isoles. *Phytochemistry*, **19**: 495-500.

van Eck, H.J., Jacobs, J.M.E., van Dijk, J., Stiekema, W.J., Jacobsen, E., 1993. Identification and mapping of three flower colour loci of potato (*S. tuberosum* L.) by RFLP analysis. *Theoretical and Applied Genetics*, **86**: 295-300.

van Eck, H.J., Jacobs, J.M.E., van den Berg, P.M.M.M., Stiekema, W.J., Jacobsen, E., 1994. The inheritance of anthocyanin pigmentation in potato (*Solanum tuberosum* L.) and mapping of tuber skin colour loci using RFLPs. *Heredity*, **73**: 410-421.

van Marle, J.T., van Dijk, C., Voragen, A.G.J., Biekman, E.S.A., 1994. Comparison of the cooking behaviour of the potato cultivars Nicola and Irene with respect to pectin breakdown and the transfer of ions. *Potato Research*, **37(2)**: 183-195.

van Sumere, C.F., 1989. Phenols and phenolic acids. In: Harborne, J.B. (Ed.). *Methods in Plant Biochemistry, Volume 1. Plant Phenolics.* Academic Press, London. pp. 29-73.

van Sumere, C.F., Cottenie, J., de Greef, J., Klint, J., 1972. Biochemical studies in relation to the possible germination regulatory role of naturally occurring coumarins and phenolics. In: Runeckles, V.C., Watkin, J.E. (Eds.). *Recent Advances in Phytochemistry, Volume 4.* Appleton-Century-Crofts, New York. pp. 165-221.

Verma, S.C., Purohit, L.K., Sharda, R.T., Purohit, A.N., Upadhya, M.D., 1972. Anthocyanin in dark- and light-grown sprouts of potato. *Potato Research*, **15(2)**: 166-169.

Vogt, E., Schönherr, J., Schmidt, H. W., 1983. Water permeabiltiy of periderm membranes isolated enzymatically from potato tubers. *Planta*, **158**: 294-301.

Vreugdenhil, D., Bindels, P., Reinhoud, P., Klocek, J., Hendriks, T., 1994. Use of the growth retardant tetcyclacis for potato tuber formation *in vitro*. *Plant Growth Regulation*, **14(3)**: 257-265.

Walker, J.R.L., 1975. *The Biology of Plant Phenolics.* Edward Arnold, London.

Walker, J.R.L., 1995. Enzymic browning in fruits: its biochemistry and its control. In: Lee, C.Y., Whitaker, T.J. (Eds.). *Symposium on Enzymic Browning and its Prevention in Foods. Symposium Series No. 600.* American Chemistry Society, Washington D. C. pp. 8-22.

Wang, P., Hu, C., 1982. *In vitro* mass tuberization and virus-free seed-potato production in Taiwan. *American Potato Journal*, **59**: 33-37.

Weidenbörner, M., Jha, H.C., 1993. Antifungal activity of flavonoids and their mixtures against different fungi occurring on grain. *Pesticide Science*, **38(4)**: 347-351.

Weissenböck, G., 1975. Changes in the activity of phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL) and chalcone-flavanone isomerase in relation to the accumulation of C-glycosylflavones in developing shoots of oat seedlings (*Avena sativa* L.) in light and darkness. *Zeitschrift fur Pflanzenphysiologie*, **74**: 226-254.

Whitehead, T., McIntosh, T.P., Findlay, W.M., 1953. *The Potato in Health and Disease.* Oliver and Boyd, Edinburgh.

Wietschel, G., Reznik,H., 1980a. The flavonoid patterns of tuber-bearing *Solanum*-species. II. The flavonoid glycosides of the species from Series I - XVI. *Zeitschrift fur Pflanzenphysiologie*, **99**: 79-88.

Wietschel, G., Reznik,H., 1980b. The flavonoid patterns of tuber-bearing *Solanum*-species. III. The flavonoid glycosides of the species from Series XVII. *Zeitschrift fur Pflanzenphysiologie*, **99**: 149-158.

Wilkinson, E.M., Butt, V.S., 1992. Enzyme changes during lignogenesis in pea shoots induced by illumination. *Journal of Experimental Botany*, **43(254)**: 1259-1265.

Wilkinson, M.J., Donnelly, A., Black, I., 1994. *The Commonwealth Potato Collection. Inventory for 1994.* Scottish Crop Research Institute, Dundee, Scotland.

Wilson, J.M., Wong, E., 1976. Peroxidase catalysed oxygenation of 4',2,4-trihydroxychalcone. *Phytochemistry*, **15**: 1333-1341.

Wrolstad, R.E., Lee, D.D., Poei, M.S., 1980. Effect of microwave blanching on the color and composition of strawberry concentrate. *Journal of Food Science*, **45**: 1573-1577.

Yamada, T., Komiya, T., Akaki, M., 1980. Formation of an inclusion complex of anthocyanin with cyclodextrin. *Agricultural and Biological Chemistry*, **44(6)**: 1411-1413.

Yatsuhashi, H., Hashimoto, T., 1985. Multiplicative action of a UV-B photoreceptor and phytochrome in anthocyanin synthesis. *Photochemistry and Photobiology*, **41(6)**: 673-680.

Yen,D.E., 1961/62. The potato of early New Zealand. *The Potato Journal*, **1961/62**: 2-5.

Zrenner, R., Salanoubat, M., Willmitzer, L., Sonnewald, U., 1995. Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). *The Plant Journal*, **7(1)**: 97-107.

Zucker, M., 1963. The influence of light on synthesis of protein and of chlorogenic acid in potato tuber tissue. *Plant Physiology*, **38**: 575-580.

Zucker, M., 1965. Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. *Plant Physiology*, **40(5)**: 779-784.

Zucker, M., 1968. Sequential induction of phenylalanine ammonia-lyase and a lyase-inactivating system in potato tuber disks. *Plant Physiology*, **43**: 365-374.

APPENDIX 1

Potato characteristics

Table A1.1 Parentage of *Solanum tuberosum* cultivars used.

Cultivar	parentage
Arran Victory	uncertain - possibly Suttons Abundance x self
Blue Derwent	unknown
Catriona	unknown - bred by A. Findlay in 1920
Desirée	Urgenta x Depesche
I29	unknown
I53	unknown
Kowiniwini	unknown
Maori Chief (I39)	selection from Northern Star
Moe Moe (I50)	unknown
M10	unknown
Ngaoutiouti (I43)	unknown
Northern Star	unknown - bred by A. Findlay in 1900
Old Red (I28)	unknown
O60/1	imported from Scotland - '*Neotuberosum*'
Poiwa (I145)	unknown
Raupi (I197)	unknown
Red Flesh	Stage II Blue x Rua
Red Rascal	Tekau x Desirée
Red Rocks (I51b)	unknown
Rua	Katahdin x Harford
Russet Burbank	sport of Burbank
Skerry Blue (I164)	unknown
Stage II Blue	Urenika x V431-14
Tekau	1584c(10) x [109-3 x 119-227]
Urenika	unknown - probably synonymous with Congo
Whitu	Dakota x [1512c(16) x 119-227]
177/3	Tekau x V394
289/3	1067-47 x V394
1858.21	Golden Wonder x 3069abc(3)
761/1	*S. stenotonum* x *S. phureja* diploid from Scotland

Note all cultivars are tetraploid (*S. tuberosum*), except 761/1 which is diploid

Table A1.2 Characteristics of *Solanum tuberosum* cultivars used.

Cultivar	Tuber - skin colour	Tuber - flesh colour	Flower colour
Arran Victory	purple (often scattered)	white, purple vas. tissue	light purple / white
Blue Derwent	medium-dark purple	white, purple vas. tissue	-
Catriona	brown with purple eyes	white	purple / white
Desirée	pink/red	yellow	light purple / white
I29	medium pink	yellow	white
I53	medium-dark purple	purple and white inside vas. ring, white ouside vas. ring	white
Kowiniwini	medium-dark purple with white spectacles	yellow	dark purple
Maori Chief (I39)	scattered purple	white	white
Moe Moe (I50)	light purple	yellow, purple vas. tissue	purple
M10	brown	yellow	white
Ngaoutiouti (I43)	light purple, scattered	yellow	purple
Northern Star	brown	white	-
Old Red (I28)	dark purple	white	-
O60/1	medium red	yellow	light purple
Poiwa (I145)	light purple	yellow-purple vas. tissue	purple / white
Raupi (I197)	brown/light purple	white	purple / white
Red Flesh	dark red	red/pink inside vas. ring, yellow outside vas. ring	mostly white / some pink
Red Rascal	pink/red	white	light purple / white
Red Rocks (I51b)	light pink	white	white
Rua	white	white	white
Russet Burbank	brown	white	white
Skerry Blue (I164)	medium purple	white, purple vas. tiissue	white / purple
Stage II Blue	dark purple/black	purple inside vas. ring, white outside vas. ring	white
Tekau	white (with purple eyes)	white	white
Urenika	dark purple/black	dark purple	white and purple
Whitu	white	white	-
177/3	brown	yellow	purple / white
289/3	white (with some purple)	yellow	purple
1858.21	white (with pink eyes)	white	white
Diploid	white	yellow	very dark purple/blue

vas. = vascular

Note for flower colour, where two colours are noted the flower is striped and the predominant colour is listed first.

Table A1.3 Characteristics of *Solanum* species and lines used

Species	line	%germ	habit	plant	skin colour	flower colour
S. acaule	ACL 2113	76	S	1	white	light purple
				7	white	white
				11	white	white
S. acaule	ACLAEM 3734	16	S	2	white	light purple
				4	white	light purple
S. berthaultii	BER 4036	8	M	1	white	dark purple
				2	white	purple / white
S. gourlayi	GRL 2480	72	T	6	white	purple
				9	light purple around eyes	purple
S. oplocense	OPL 3777	32	M	4	white	purple
				8	white	light purple
S. sanctae-rosae	SCT 3269	88	S	2	white	dark purple
				17	white	dark purple
S. santae-rosae	SCT 3779	52	S	6	light purple	white
				12	white	purple
				13	light purple around eyes	purple / white
S. sparsipilum	SPL 3488	76	M	2	light purple around eyes	purple
				6	light purple around eyes	purple
S. sparsipilum	SPL 3563	60	M-T	2	light purple around eyes	dark purple
				3	light purple around eyes	dark purple
S. speggazzinii	SPG 3745	60	T	6	some light purple	white
				7	light purple	white / light purple
S. speggazzinii	SPG 3791	72	T	2	some light purple	white / light purple
				15	some light purple	white / light purple
S. stenotomum	STN 4711	48	T	5	white	white / light purple
				12	white	white / light purple

%germ:- percentage germination of imported seeds

habit:- S = small, M = medium, T = tall

plant:- plant number assigned by NZ Institute for Crop and Food Research, Lincoln

APPENDIX 2

Retention times and chromatographic properties of selected standards.

Compound	Ret time	Rf (a)	Rf (b)	Colour of spot (1)	(2)	(3)	(4)	(5)	λ_{max}(nm)	
Benzoic acids										
gallic acid	3.4	60.5	53.8	-	dp	fbl/p	fbl/p	p	272	
gentisic acid	6.5	92.1	63.1	-	fgr	fbl/gr	fbl	fgr	328	
p-hydroxybenzoic acid	6.7	ND	ND	ND	ND	ND	ND	ND	256	
protocatechuic acid	4.2	83.8	65.6	-	dp	fbl	fbl/p	dp	260	294
salicylic acid	21.8	96.0	78.1	-	fbl	fbl	dp	dp	302	
syringic acid	12.9	88.0	68.1	-	dp	dp	dp	dp	276	
vanillic acid	12.6	90.5	71.9	-	dp	dp	dp	dp	262	292
Cinnamic acids										
caffeic acid	9.3	77.2	35.6	-	fbl/p	fbl/p	fbl	fbl	302sh	322
chlorogenic acid	7.8	61.9	65.8	-	fbl	fgr	fgr	fgr	302sh	326
t-cinnamic acid	30.9	ND	ND	ND	ND	ND	ND	ND	280	
p-coumaric acid	15.3	92.8	49.4	-	dp	fbl	fbl/p	fbl	302sh	310
ferulic acid	18.9	84.6	43.1	-	fbl	fbl	fbl	fbl	298sh	322
sinapic acid	20.0	79.2	38.1	-	fbl/gr	fbl/gr	dbl	fbl/gr	322	
Flavonols										
kaempferol	35.8	83.2	1.9	sl y	fo	fo	fy	fo	266	366
Km-3-rut	24.7	60.1	60.6	-	dp	fy	fy	fy	266	346
myricetin	25.3	37.9	0.0	sl o	fo	fo	fo	pk/r	254	370
quercetin	30.6	62.9	1.2	sl y	fo	fy/o	fo	fo	256	366
Qu-3-gal	21.1	ND	ND	ND	ND	ND	ND	ND	256	362
Qu-3-glu	21.9	59.4	29.7	-	dp	fo	fo	fo/y	256	352
Qu-3-rha	24.4	ND	ND	ND	ND	ND	ND	ND	256	350
Qu-3-xyl	22.5	ND	ND	ND	ND	ND	ND	ND	256	352
rutin	21.7	46.7	47.5	-	dp	fo/br	fo	fo	256	352
Flavan-3-ols										
catechin	5.2	63.8	54.4	-	dp	fbl	dp	fbl	280	
epicatechin	10.4	54.4	49.4	-	dp	fbl	dp	fbl	280	
Flavanones										
eriodictyol	26.8	90.0	35.0	-	dp	dp	fo/pk	dp	286	324sh
homoeriodictyol	30.7	ND	ND	ND	ND	ND	ND	ND	288	
naringenin	32.0	96.8	42.5	-	dp	dp	fgr/y	fo	290	324sh
Dihydroflavonols										
dihydrokaempferol	20.5	ND	ND	ND	ND	ND	ND	ND	290	330sh
dihydromyricetin	7.6	65.6	44.3	-	dp	dp	dp	dp	292	330sh
dihydroquercetin	15.5	83.8	54.4	-	dp	dp	br	dp	288	324sh
Flavones										
apigenin	35.5	92.8	4.4	-	dp	br	fy	fy	266	338
luteolin	31.1	77.2	1.9	-	dp	fgr/y	fy	fy/br	254	348
luteolin-7-glucoside	21.0	ND	ND	ND	ND	ND	ND	ND	266	344
Anthocyanins										
Cy-3-gal	18.4	ND	ND	ND	ND	ND	ND	ND		526
Cy-3-glu	17.7	ND	ND	ND	ND	ND	ND	ND		526
Cy-3-rut	20.7	41.8	53.1	r/p	dp	dp	dp	dp		526
Dp-3-rut	17.2	23.4	16.3	p	dp	dp	dp	dp		536
Mv-3-glu	24.2	41.8	41.3	p	dp	bl/p	dp	dp		538
Pg-3-rut	23.0	48.1	63.8	o/pk	dp/r	dp/r	r	y/br		510

Appendix 2 continued...

Compound	Ret time	Rf (a)	Rf (b)	Colour of spot					λ_{max}(nm)	
				(1)	(2)	(3)	(4)	(5)		
Coumarins										
aesculetin	7.9	ND	ND	ND	ND	ND	ND	ND	296	342
4-hydroxycoumarin	21.8	ND	ND	ND	ND	ND	ND	ND	282	298sh
scopoletin	17.6	ND	ND	ND	ND	ND	ND	ND	294	342
Proanthocyanidins										
procyanidin B2	6.2	ND	ND	ND	ND	ND	ND	ND	ND	
procyanidin B5	18.8	ND	ND	ND	ND	ND	ND	ND	ND	

Key to colours

d = dull
f = fluorescent
sl = slightly
bl = blue
br = brown
gr = green
o = orange
p = purple
pk = pink
r = red
y = yellow

Abbreiviations

Sugars

rut - rutinoside
glu - glucoside
gal - galactoside
rha - rhamnoside
xyl - xyloside

Flavonoids

Pg - pelargonidin
Cy - cyanidin
Dp - delphinidin
Mv - malvidin
Km - kaempferol
Qu - quercetin

λ_{max} (nm) = wavelength (nm) of maximum absorbance
- = no colour
ND = not determined
Ret time = Retention time for analytical C_{18} HPLC, see Chapter 3 for method
(a) Rf (x 100) BAW
(b) Rf (x100) 15% HOAc
(1) Visible colour of spot
(2) Colour of spot under UV light
(3) Colour of spot under UV light after exposed to NH_3 fumes
(4) Colour of spot after spraying with NA reagent, viewed under UV light
(5) Colour of spot after spraying with Na carbonate, viewed under UV light

APPENDIX 3

Concentration (μg/gFW) of phenolic acids, flavonoids and anthocyanins in *Solanum tuberosum* cultivars

Key

Phenolic acids

gal	gallic acid
pro	protocatechuic acid
p-ben	*p*-hydroxybenzoic acid
chl	chlorogenic acid
caf	caffeic acid
UP1	unknown phenolic acid 1
van	vanillic acid
syr	syringic acid
coum	*p*-coumaric acid
fer	ferulic acid
UP2	unknown phenolic acid 2
sin	sinapic acid
sal	salicylic acid
UP3	unknown phenolic acid 3
cinn	cinnamic acid

Flavonoids

cat	catechin
epicat	epicatechin
2a	flavonoid 2a (quercetin-glucose-rhamnose)
2b	flavonoid 2b (quercetin-glucose-rhamnose)
UF1	unknown flavonoid 1
q-3-gal	quercetin-3-galactoside
q-3-glu	quercetin-3-glucoside
eriod	eriodictyol
Km-3-r	kaempferol-3-rutinoside
My	myricetin
UF2	unknown flavonoid 2
narin	naringenin

Anthocyanins

UA1 (14.2)	unknown anthocyanin 1 - retention time 14.2min
UA2 (15.8)	unknown anthocyanin 2 - retention time 15.8min
Dp-3-r	delphinidin-3-rutinoside
Cy-3-r	cyanidin-3-rutinoside
UA3 (23.0)	unknown anthocyanin 3 - retention time 23.0min
Pg-3-r	pelargonidin-3-rutinoside
Mv-3-r	malvidin-3-rutinoside
anth-1	petunidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside
Pn-gly (25.3)	peonidin-glycoside - retention time 25.3min
anth-3	pelargonidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside
anth-2	malvidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside
UA4 (28.0)	unknown anthocyanin 4 - retention time 28.0min
UA5 (34.2)	unknown anthocyanin 5 - retention time 34.2min

*	1992/93 season
**	1993/94 season
flowers[1]	plants that did not flower, or did not produce enough flowers for analysis
flavonoids[x]	sum of total flavonoids excluding myricetin and UF2, as in a number of cultivars reliable concentrations of these compounds could not be obtained (see 'a'), so all totals were comparable
a	due to co-elution of myricetin and UF2 with anthocyanins, reliable concentrations of these could not be determined
-	not detected (*i.e.* concentration equals zero)
Wfl/wsk	white flesh/white skin
Wfl/colsk	white flesh/coloured skin (red or purple)
Purfl/pursk	purple flesh/purple skin
Redfl/redsk	red flesh/red skin

Table A 3.1 Phenolic acids

Cultivar		gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total phenolic acids
Arran Victory	* skin	9.6	420.2	-	2011.9	174.2	63.2	85.2	10.5	17.8	18.7	20.4	39.3	12.9	17.5	1.9	2785.3
	* flesh	5.1	202.0	-	340.6	1.4	51.0	-	-	23.0	0.7	13.2	1.4	-	-	-	638.6
	** skin	10.4	258.6	-	2689.8	579.5	173.0	40.0	29.5	6.9	13.6	35.2	29.5	13.1	-	-	3879.1
	** flesh	3.3	167.2	-	133.5	10.2	8.6	14.2	0.7	2.2	1.1	2.5	2.1	-	-	-	345.6
	* flowers[1]																
	* leaves	14.5	48.0	-	479.0	4.6	104.2	-	7.4	3.3	4.0	13.1	9.9	-	4.5	0.5	693.0
Blue Derwent	* skin	11.8	516.0	-	2697.4	145.1	81.5	99.9	15.3	15.1	26.7	27.5	89.6	25.5	-	3.0	4393.0
	* flesh	6.1	84.6	-	162.2	-	28.5	-	-	5.2	0.5	2.3	2.7	-	-	-	292.1
	** skin	7.9	372.6	-	3680.8	445.3	124.5	78.4	16.5	3.3	10.8	9.7	14.6	10.8	15.5	5.2	4809.9
	** flesh	1.6	61.7	-	157.6	2.8	28.2	-	-	-	1.6	1.9	-	-	-	-	255.4
	* flowers[1]																
	* leaves	9.8	26.1	21.0	255.2	-	41.9	-	-	1.6	1.1	5.5	4.5	-	2.1	-	368.8
Catriona	* skin	9.6	325.0	-	1192.7	83.9	33.4	72.0	3.5	2.7	7.4	15.6	20.7	11.4	6.2	1.7	1784.1
	* flesh	5.2	73.0	-	38.9	-	6.6	-	-	9.2	-	2.3	4.5	-	-	-	139.7
	** skin	13.0	314.3	-	2279.3	294.0	134.8	35.5	13.7	6.0	11.0	20.7	39.3	10.8	13.9	3.7	3220.0
	** flesh	2.4	76.9	-	22.8	3.0	5.2	-	-	-	-	4.4	1.0	-	-	-	115.7
	* flowers	4.3	27.9	-	802.4	3.5	21.4	-	2.4	5.6	6.7	7.6	3.7	-	5.5	0.4	902.4
	* leaves	21.8	86.9	95.2	512.9	5.5	49.6	-	6.6	9.2	10.4	6.8	18.4	-	-	-	823.3

Table A3.1 Phenolic acids continued...

Cultivar		gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total phenolic acids
Desirée	* skin	8.4	311.6	-	1128.7	31.3	378.6	98.4	11.4	27.7	23.2	17.8	75.9	89.3	-	-	2292.3
	* flesh	6.7	258.5	-	28.3	-	7.1	4.5	-	27.6	-	4.8	-	-	-	-	337.5
	** skin	-	295.2	-	1862.8	223.1	126.8	168.3	11.2	10.1	25.2	48.2	107.6	44.8	-	-	2923.3
	** flesh	-	97.4	-	159.5	-	10.1	-	-	-	2.5	2.9	1.1	-	-	-	273.5
	* flowers	4.6	17.7	-	653.3	4.1	15.7	8.5	2.0	-	13.5	6.4	2.8	-	4.1	-	732.7
	* leaves	14.8	38.7	-	160.1	11.9	26.7	15.9	5.1	1.7	2.8	10.4	18.1	-	3.9	0.9	311.0
I29	* skin	9.4	402.1	-	2194.8	122.3	403.5	62.3	8.4	5.7	17.5	14.1	143.4	34.8	12.0	1.8	3432.1
	* flesh	4.4	100.5	-	66.9	1.1	11.2	5.7	-	5.2	0.5	4.9	1.5	-	-	-	201.9
	** skin	-	368.2	-	2019.0	403.3	166.6	121.6	15.1	7.8	24.1	34.2	26.8	25.9	44.0	-	3256.6
	** flesh	3.3	85.1	-	334.2	3.8	29.1	8.4	-	1.9	4.0	4.1	1.1	-	5.5	-	480.5
	* flowers	4.3	67.1	10.5	774.3	4.7	18.9	5.0	1.9	1.9	5.6	13.8	2.9	-	-	-	910.9
	* leaves	19.4	99.5	104.2	756.3	7.9	127.2	-	6.3	5.1	3.7	12.4	8.4	-	4.3	0.2	1142.5
I53	* skin	16.7	401.8	-	1632.4	139.6	26.4	52.9	4.6	10.9	10.0	12.7	10.9	11.0	8.5	-	2338.4
	* flesh	8.9	114.0	-	258.0	1.2	11.8	20.5	-	23.9	0.8	4.8	3.1	-	-	-	447.0
	** skin	11.8	393.3	-	3290.9	564.8	111.6	178.8	35.5	12.2	10.6	33.7	44.4	9.2	-	3.9	5150.7
	** flesh	-	88.0	-	285.2	6.2	27.5	38.0	-	1.0	1.5	7.9	1.1	-	1.9	-	458.3
	* flowers	5.1	14.4	8.1	608.2	2.3	16.3	7.2	3.8	-	15.9	11.2	-	-	6.1	-	698.6
	* leaves	11.1	52.0	98.2	324.6	23.3	55.7	11.4	4.3	3.9	9.5	-	8.7	-	-	-	602.7

Table A3.1 Phenolic acids continued...

Cultivar		gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total phenolic acids
Kowiniwini	* skin	9.6	187.3	-	1285.0	158.4	123.6	86.8	-	5.7	10.9	16.5	135.4	16.8	-	-	2036.0
	* flesh	5.3	119.6	-	9.2	-	8.8	2.6	-	31.7	-	4.5	4.0	-	-	-	185.7
	** skin	13.9	167.4	-	1315.6	225.6	44.8	257.9	7.1	7.8	12.6	47.6	11.6	10.7	14.7	3.8	2141.1
	** flesh	-	98.7	-	45.9	4.4	8.2	6.5	-	-	2.1	5.2	-	-	-	-	171.0
	* flowers[1]																
	* leaves	14.9	60.1	104.7	404.5	27.2	54.8	-	19.3	3.8	14.3	13.5	9.8	-	-	-	726.9
Maori Chief	* skin	12.0	227.5	-	847.0	96.3	255.6	40.6	11.1	10.8	20.7	13.0	233.6	27.4	13.7	-	1609.3
	* flesh	5.9	94.6	-	59.8	0.8	15.5	-	-	9.5	-	1.7	3.7	-	-	-	191.5
	** skin	-	387.3	-	976.4	246.4	209.9	52.3	30.8	10.2	6.4	25.1	17.4	25.9	14.8	2.5	2050.4
	** flesh	6.1	103.8	-	63.2	0.9	16.4	-	-	8.9	-	1.6	3.9	-	-	-	204.8
	* flowers[1]																
	* leaves	9.0	21.1	17.5	153.5	-	44.8	-	0.8	2.8	1.7	2.5	3.3	-	3.6	-	260.6
Moe Moe	* skin	8.9	267.5	-	1757.2	159.4	52.6	58.7	6.2	2.1	15.1	8.4	27.4	7.9	13.6	1.5	2386.5
	* flesh	5.4	54.4	-	78.1	1.3	21.5	-	-	2.4	1.6	2.2	4.0	-	-	-	168.7
	** skin	-	325.3	-	2041.9	280.7	105.2	111.6	14.0	10.2	9.8	43.2	28.0	6.3	-	3.2	2989.4
	** flesh	-	61.6	-	57.8	5.4	9.5	4.0	-	3.6	3.5	3.9	-	-	-	-	149.3
	* flowers	4.8	32.4	-	986.1	5.4	16.3	4.4	2.6	1.8	10.5	10.5	1.8	-	3.8	0.7	1081.1
	* leaves	14.2	36.1	65.5	382.2	-	37.6	-	0.8	1.8	4.0	19.0	2.3	-	3.1	-	566.6

Table A3.1 Phenolic acids continued...

Cultivar		gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total phenolic acids
M10	* skin	8.7	215.2	-	746.4	52.0	29.4	183.0	3.0	2.2	5.2	4.4	35.2	9.0	1.8	2.1	1297.6
	* flesh	7.0	199.9	-	28.0	-	6.6	6.5	-	26.2	1.4	10.4	-	-	-	-	289.3
	* flowers	5.4	12.4	6.5	471.4	4.7	19.8	-	4.3	-	4.1	14.5	-	-	5.8	0.7	549.6
	* leaves	10.1	27.1	42.2	48.5	-	15.4	4.7	0.7	1.8	2.0	4.6	1.6	-	2.8	-	161.5
Ngaoutiouti	* skin	6.6	211.7	-	1290.9	84.7	29.6	56.5	2.0	5.3	9.6	3.7	26.1	5.1	-	1.2	1733.0
	* flesh	7.9	59.5	-	56.3	-	11.8	7.0	-	4.0	1.5	5.3	2.8	-	-	-	156.1
	** skin	10.4	221.4	-	2175.9	292.0	94.1	75.3	4.6	5.6	8.7	17.9	20.1	4.2	-	2.7	2932.9
	** flesh	-	54.8	-	65.6	5.7	10.3	5.1	0.7	-	2.2	5.8	-	-	-	-	150.2
	* flowers	6.0	78.1	-	892.8	4.0	19.3	5.3	3.1	-	9.9	12.8	-	-	4.5	1.0	1036.8
	* leaves	18.5	91.7	127.4	711.7	9.7	85.1	-	6.9	3.0	7.1	40.3	6.5	-	8.0	0.7	1116.6
Northern Star	* skin	9.6	227.9	-	748.0	95.7	43.5	192.8	10.5	2.7	11.0	4.2	132.6	11.8	4.4	2.8	1497.5
	* flesh	5.7	33.0	-	48.4	1.5	13.5	-	-	0.6	-	-	2.9	-	-	-	105.6
	** skin	-	250.6	-	2139.1	272.8	50.1	77.1	25.1	11.5	10.5	15.1	19.1	8.6	26.7	2.6	2908.9
	** flesh	1.9	46.9	-	48.0	1.5	16.5	5.2	-	-	1.1	1.4	-	-	-	-	122.5
	* flowers[1]																
	* leaves	12.6	37.4	48.9	301.0	0.6	61.6	-	1.7	3.9	2.4	2.7	5.9	-	2.8	0.8	482.3

Table A3.1 Phenolic acids continued...

Cultivar		gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total phenolic acids
Old Red	* skin	9.6	242.1	-	1472.7	110.2	42.5	242.1	8.7	17.2	11.3	13.7	131.8	17.3	41.5	-	2360.7
	* flesh	7.7	109.0	-	14.8	-	4.7	7.1	-	10.3	-	3.0	4.2	-	-	-	160.8
	** skin	9.7	265.3	-	2083.6	66.8	47.7	100.7	4.2	16.5	11.0	53.4	13.0	12.9	30.1	5.7	2720.6
	** flesh	-	144.2	-	22.5	3.9	1.9	13.6	-	-	3.2	5.6	-	-	-	-	194.9
	* flowers[1]																
	* leaves	17.9	53.9	72.6	150.8	10.4	67.4	-	6.1	6.0	5.5	13.3	8.1	-	2.8	-	444.8
O60/1	* skin	10.2	264.0	-	1605.1	213.8	30.9	61.9	8.8	7.0	14.7	4.1	42.0	19.1	26.4	1.8	2309.8
	* flesh	4.4	105.0	-	156.5	2.0	13.2	44.5	-	0.8	-	3.0	7.8	-	-	-	337.2
	** skin	23.2	231.8	-	2417.2	518.7	87.5	108.7	8.4	7.0	8.7	38.4	9.3	17.3	29.5	5.8	3311.5
	** flesh	3.3	81.4	-	161.7	6.3	11.1	12.8	-	0.5	-	4.3	1.9	-	-	-	283.3
	* flowers	8.5	64.9	8.2	750.1	5.2	24.6	-	2.1	6.8	8.0	6.1	8.0	-	5.2	0.4	898.1
	* leaves	16.7	51.0	70.2	512.5	3.5	162.1	-	-	8.4	11.8	8.8	-	-	-	0.3	845.3
Poiwa	* skin	7.2	259.4	-	1345.0	112.0	39.8	101.3	4.0	1.3	13.7	6.0	28.5	6.7	7.7	1.2	1933.8
	* flesh	-	84.7	-	34.1	1.5	5.6	6.6	-	9.0	1.3	-	4.8	-	-	-	147.6
	** skin	10.3	185.4	-	1286.0	193.8	45.4	28.2	2.0	4.0	8.0	17.7	12.6	5.2	-	2.3	1791.9
	** flesh	3.0	49.1	-	41.5	3.8	9.8	5.8	-	-	-	6.2	-	-	-	-	119.2
	* flowers	4.6	44.2	7.4	900.4	5.6	16.7	6.1	3.1	-	11.3	9.9	-	-	4.3	0.8	1014.4
	* leaves	18.1	35.6	94.2	212.0	17.6	32.9	-	-	8.6	5.6	23.5	6.8	-	-	-	454.9

Table A3.1 Phenolic acids continued...

Cultivar		gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total phenolic acids
Raupi	* skin	8.4	221.3	-	521.4	80.5	50.4	51.8	6.7	4.1	11.4	5.0	5.1	9.7	-	4.4	975.8
	* flesh	-	53.4	-	30.8	-	7.2	14.9	-	3.1	-	-	2.6	-	-	-	126.0
	** skin	11.4	287.8	-	1277.0	312.3	59.8	33.0	2.0	5.3	13.1	11.0	7.3	7.2	-	3.3	2030.5
	** flesh	-	59.5	-	23.0	5.4	5.8	16.5	-	3.6	2.9	3.6	-	-	-	-	120.3
	* flowers	4.6	34.5	-	1037.8	4.8	23.8	-	3.6	-	8.7	9.7	5.3	-	4.6	0.2	1127.9
	* leaves	18.2	84.6	91.3	581.4	-	70.6	-	2.2	3.5	2.9	19.0	3.2	-	3.1	0.4	880.4
Red Flesh	* skin	8.2	285.7	44.4	2234.3	99.2	100.8	76.9	-	16.6	64.7	52.8	54.8	65.3	120.7	14.7	3239.1
	* flesh	5.0	125.6	-	486.4	-	34.0	47.8	-	15.9	7.2	5.8	1.6	6.5	-	0.3	736.1
	** skin	-	262.6	42.7	2660.0	327.9	191.2	108.6	-	9.5	60.0	63.9	76.6	63.7	64.5	8.5	3889.7
	** flesh	1.9	75.8	-	408.2	9.7	48.3	47.5	-	-	17.2	2.1	-	4.3	13.6	-	628.6
	* flowers	4.7	16.8	6.2	789.6	4.9	56.9	-	1.8	-	0.6	7.5	3.1	-	2.5	0.5	895.1
	* leaves	15.0	54.9	99.9	497.4	7.7	277.7	-	10.8	3.9	5.2	10.3	6.9	-	8.9	-	998.6
Red Rascal	* skin	5.8	328.1	-	1657.0	58.1	33.4	70.2	-	6.5	17.5	6.0	3.5	37.5	49.6	-	2273.2
	* flesh	5.0	179.9	-	116.5	0.5	12.6	-	0.5	18.5	1.3	7.4	1.5	-	1.6	-	345.3
	** skin	-	291.1	-	2143.5	209.0	49.8	64.8	3.2	3.4	5.5	29.4	8.9	33.8	34.8	3.4	2880.6
	** flesh	2.1	105.3	-	95.1	3.1	12.0	4.2	1.3	1.4	2.0	2.9	-	-	6.4	-	235.8
	* flowers	4.6	8.7	5.1	680.8	4.1	20.4	-	3.3	-	10.9	5.4	2.3	-	4.5	0.3	750.4
	* leaves	19.6	90.3	-	1268.4	14.1	138.5	-	8.1	6.0	7.9	28.2	6.5	-	8.4	-	1596.0

Table A3.1 Phenolic acids continued...

Cultivar		gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total phenolic acids
Red Rocks	* skin	11.8	317.8	-	1222.2	109.4	154.0	82.7	2.4	15.6	12.6	88.6	122.9	22.0	7.8	-	2169.8
	* flesh	7.8	106.0	-	96.8	-	20.6	8.8	-	7.1	-	6.7	1.6	-	-	-	255.4
	** skin	18.5	162.6	-	1725.4	170.4	39.4	245.5	4.5	4.1	9.0	83.5	14.3	21.2	22.8	3.6	2514.8
	** flesh	-	47.9	-	42.0	2.8	5.7	7.3	-	-	-	5.6	-	-	-	-	111.3
	* flowers	5.2	16.1	-	902.1	3.7	17.7	5.4	3.2	-	6.6	12.3	-	-	8.8	0.3	981.4
	* leaves	17.3	48.6	69.8	515.8	6.1	109.1	-	5.1	3.7	4.7	9.4	-	-	8.0	-	797.6
Rua	* skin	6.8	160.6	-	1603.8	143.9	87.5	-	2.8	3.8	7.9	2.0	3.0	9.3	2.7	0.6	2034.7
	* flesh	6.2	44.6	-	27.8	0.9	6.7	4.5	-	0.5	-	-	-	-	-	-	91.2
	** skin	-	216.2	-	1990.3	299.6	57.5	6.4	-	2.9	7.1	8.7	27.6	8.2	11.8	7.0	2643.3
	** flesh	-	44.8	-	27.9	3.3	6.5	7.2	-	-	1.8	4.2	-	-	-	-	95.7
	* flowers	4.8	31.9	-	715.5	5.2	15.2	4.8	3.1	4.2	9.1	9.3	-	-	4.8	2.2	810.1
	* leaves	14.9	66.9	55.9	585.1	-	67.6	-	4.0	1.7	2.4	26.4	-	-	4.0	0.5	829.4
Russet Burbank	* skin	9.0	245.6	-	904.6	105.4	49.2	51.7	-	5.3	5.5	3.0	19.8	8.1	1.8	6.2	1415.2
	* flesh	6.9	180.3	-	18.2	1.0	5.1	1.9	-	22.9	-	6.8	-	-	-	-	243.1
	** skin	13.1	223.5	-	1326.9	245.7	96.5	90.7	3.1	2.8	9.5	91.7	13.5	7.6	12.0	3.2	2044.9
	** flesh	1.8	69.8	-	33.2	3.9	8.2	4.1	0.6	-	2.8	5.3	-	-	-	-	129.7
	* flowers	5.6	41.9	-	716.0	3.9	20.2	-	2.0	3.8	4.8	3.5	3.9	-	7.6	0.4	813.6
	* leaves	10.4	23.9	-	131.2	-	33.7	-	1.3	2.0	0.8	9.1	3.8	-	-	-	216.2

Table A3.1 Phenolic acids continued...

Cultivar		gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total phenolic acids
Skerry Blue	* skin	18.4	283.3	-	1697.0	104.8	276.8	40.3	-	9.2	19.3	20.3	223.5	20.8	-	2.7	2716.4
	* flesh	5.1	85.8	-	151.5	-	29.4	-	-	4.2	0.7	8.2	3.1	-	-	-	288.0
	** skin	12.5	311.5	-	2544.3	372.2	86.2	79.7	33.6	7.6	10.1	56.2	23.0	21.9	-	2.7	3561.5
	** flesh	1.8	44.9	-	107.0	2.3	15.3	-	-	-	0.9	0.9	-	-	-	-	173.1
	* flowers[1]																
	* leaves	18.7	84.4	91.3	620.6	5.0	138.6	-	6.6	5.6	5.3	4.5	11.6	-	4.6	-	996.8
Stage II Blue	* skin	5.0	194.9	-	2337.9	314.6	39.7	15.9	1.4	16.8	10.0	6.8	37.1	20.4	30.1	6.1	3036.7
	* flesh	4.9	107.3	-	254.2	-	16.6	2.7	-	0.4	2.0	3.7	4.6	-	-	-	396.4
	** skin	-	202.7	-	4766.1	1026.6	62.0	26.8	0.8	7.4	9.3	30.9	38.2	18.2	50.7	-	6239.7
	** flesh	1.6	75.8	-	359.0	7.0	40.7	26.0	-	-	-	4.9	1.0	-	-	-	516.0
	* flowers	6.4	7.6	9.0	595.2	3.6	56.7	-	3.0	-	8.0	3.9	7.2	-	5.3	0.3	706.2
	* leaves	20.4	77.4	-	804.2	7.2	283.6	-	7.9	3.9	5.5	-	9.3	-	8.4	0.5	1228.3
Tekau	* skin	9.0	232.4	-	841.0	36.5	33.2	-	2.2	2.8	8.5	7.7	3.4	15.6	37.9	-	1230.2
	* flesh	4.9	89.7	-	28.4	-	7.2	6.2	-	17.5	-	10.1	3.6	-	-	0.7	168.3
	** skin	8.2	209.7	-	1501.3	195.9	56.6	11.3	1.3	2.1	5.0	22.6	26.1	14.8	11.2	3.2	2069.3
	** flesh	2.4	53.4	-	61.4	3.0	18.9	-	-	-	-	3.5	2.5	-	-	-	145.1
	* flowers	5.8	21.2	29.0	706.4	3.5	19.8	-	2.6	4.9	5.3	3.7	2.3	-	19.6	0.4	824.5
	* leaves	13.7	43.5	-	1005.0	6.8	95.7	-	-	6.8	7.1	20.4	5.0	-	6.2	0.8	1211.0

Table A3.1 Phenolic acids continued...

Cultivar		gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total phenolic acids
Urenika	* skin	8.0	141.7	-	2784.2	595.3	81.4	51.5	5.9	36.2	32.9	23.8	27.3	39.1	-	1.4	3828.7
	* flesh	5.3	122.7	-	931.4	-	55.6	76.4	2.9	8.6	2.8	5.5	3.2	5.6	-	1.0	1221.0
	** skin	10.9	135.5	-	4039.8	726.9	184.3	87.5	2.1	11.1	5.7	77.0	23.8	35.2	-	4.6	5344.4
	** flesh	5.5	79.6	-	878.7	19.3	45.3	141.3	4.5	-	10.0	38.5	-	4.9	-	1.4	1229.0
	* flowers	3.0	42.5	4.6	659.5	2.4	45.6	-	1.1	-	2.7	3.2	-	-	2.6	-	767.2
	* leaves	10.4	23.7	16.4	203.1	0.6	138.5	-	1.2	-	1.2	3.6	1.7	-	3.0	0.3	403.7
Whitu	* skin	10.0	123.8	-	539.8	37.5	42.7	233.4	3.5	5.2	5.8	2.8	35.2	4.9	-	-	1044.6
	* flesh	4.4	79.0	-	76.2	-	14.9	7.6	-	3.5	-	1.5	1.4	-	-	-	188.5
	* flowers[1]																
	* leaves	18.5	66.9	61.0	580.3	1.4	29.0	-	2.9	2.9	4.5	-	11.7	-	3.9	-	783.0
177/3	* skin	5.1	122.2	-	557.0	71.8	73.9	39.7	1.9	2.9	7.3	3.9	28.6	7.7	13.1	-	935.1
	* flesh	7.9	42.8	-	13.4	-	2.0	-	-	3.3	-	3.2	-	-	-	-	72.6
	** skin	7.2	135.1	-	1235.2	140.7	63.4	16.2	7.8	5.8	12.3	5.5	20.8	-	27.7	2.0	1679.7
	** flesh	1.9	34.2	-	25.4	2.9	5.3	-	-	-	3.2	4.6	-	-	-	-	77.5
	* flowers	6.4	48.9	7.3	203.2	4.3	18.8	-	1.9	-	8.9	7.0	3.9	-	5.5	0.4	316.5
	* leaves	29.7	12.9	-	1173.2	8.1	153.2	-	11.0	12.7	14.4	-	-	-	-	0.8	1416.0

Table A3.1 Phenolic acids continued...

Cultivar		gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total phenolic acids
289/3	* skin	12.2	466.6	-	1059.0	122.7	26.7	36.2	9.7	4.4	11.0	22.9	22.0	-	5.0	3.9	1802.3
	* flesh	6.3	119.7	-	73.7	1.5	17.4	-	-	15.0	1.5	27.2	7.5	-	-	-	269.8
	** skin	10.9	298.6	-	1745.2	230.0	111.0	12.8	4.8	7.4	23.6	28.1	29.6	-	7.5	2.5	2512.0
	** flesh	-	63.3	-	66.7	2.6	11.8	4.3	-	0.8	1.4	3.9	-	-	-	-	154.8
	* flowers	7.1	35.9	7.2	599.0	2.4	15.6	4.6	2.7	-	5.9	7.5	5.8	-	4.6	0.3	698.6
	* leaves	13.5	30.6	-	452.5	4.5	55.2	-	-	3.4	1.8	-	7.1	-	1.8	-	570.4
1858.21	* skin	5.7	107.1	-	494.7	54.2	42.7	63.0	1.1	3.6	4.9	2.3	21.3	6.8	1.8	7.9	817.1
	* flesh	5.0	50.5	-	67.2	-	18.3	-	-	1.4	-	-	-	-	-	-	142.4
	** skin	-	139.3	-	1069.8	152.4	85.8	21.7	4.4	2.7	3.1	10.4	14.2	2.0	15.0	3.0	1543.8
	** flesh	-	34.6	-	82.8	1.7	13.7	2.6	-	-	0.5	4.2	-	-	-	-	140.1
	* flowers	4.2	11.2	-	551.6	2.1	29.8	66.1	1.3	-	5.5	10.0	2.3	-	4.4	0.8	689.3
	* leaves	13.6	22.6	36.4	390.1	-	38.0	-	-	3.3	0.9	4.0	3.2	-	-	-	512.1
Diploid	* skin	5.5	310.6	27.6	945.4	14.8	62.6	51.5	-	8.1	3.6	3.0	21.5	3.8	2.5	-	1432.9
	* flesh	-	137.8	-	31.7	-	11.0	-	-	12.9	-	2.0	2.0	-	-	-	197.4
	* flowers	4.2	31.8	5.6	727.9	2.7	0.8	2.1	-	2.0	4.2	3.4	1.9	-	17.2	0.3	804.1
	* leaves	15.8	37.3	107.9	542.1	5.9	77.0	-	3.3	11.4	4.0	14.0	7.8	-	-	0.3	826.8

Table A3.2 Flavonoids

Cultivar		cat	epicat	2a	2b	UF1	q-3-gal	rutin	q-3-glu	eriod	Km-3-r	My	UF2	narin	total flavonoids[x]
Arran Victory	* skin	62.0	35.1	20.0	10.6	5.9	-	-	-	56.9	29.3	a	a	69.8	289.6
	* flesh	10.8	9.6	-	4.5	-	-	-	-	0.8	1.4	a	a	-	27.1
	** skin	94.3	19.6	13.3	12.0	6.7	-	-	-	29.8	-	a	a	151.1	353.9
	** flesh	19.6	-	-	7.9	-	-	-	-	-	1.5	a	-	0.9	29.9
	* flowers[1]														
	* leaves	25.7	31.3	464.3	491.5	-	12.5	644.5	11.3	6.7	20.1	4.8	2.7	2.8	1710.7
Blue Derwent	* skin	82.9	52.9	25.4	7.0	10.1	-	-	-	46.1	-	a	a	65.5	289.9
	* flesh	7.0	-	-	-	-	-	-	-	2.2	-	-	-	-	9.2
	** skin	74.4	31.7	10.1	6.3	6.6	-	-	-	16.2	-	a	a	97.4	242.7
	** flesh	7.4	-	-	-	-	-	-	-	-	-	a	-	-	7.4
	* flowers[1]														
	* leaves	7.7	-	187.6	157.9	-	7.1	257.0	1.9	3.1	15.2	-	-	-	637.5
Catriona	* skin	59.6	46.3	20.5	4.6	-	-	-	-	53.3	17.8	3.4	15.7	4.9	207.0
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	113.2	101.4	6.9	13.7	-	-	-	-	27.3	11.6	a	a	57.2	331.3
	** flesh	3.8	-	-	-	-	-	-	-	0.9	-	-	-	-	4.7
	* flowers	43.4	10.2	19.8	21.9	19.4	20.9	1356.4	4.5	3.3	53.7	10.8	12.8	1.1	1554.6
	* leaves	45.5	34.5	15.8	9.1	4.4	4.0	883.5	9.8	14.1	40.9	6.0	6.1	7.4	1069.0

Table A3.2 Flavonoids continued...

Cultivar		cat	epicat	2a	2b	UF1	q-3-gal	rutin	q-3-glu	eriod	Km-3-r	My	UF2	narin	total flavonoids[x]
Desirée	* skin	62.8	83.7	22.7	-	1.8	-	-	-	2.6	114.1	a	a	49.2	336.9
	* flesh	15.3	-	-	-	-	-	-	-	-	-	-	-	-	15.3
	** skin	100.4	42.6	24.4	-	3.5	-	-	-	25.6	89.2	a	-	106.6	392.3
	** flesh	5.3	-	4.9	-	-	-	-	-	-	-	-	-	-	10.2
-	* flowers	-	15.1	1477.2	94.1	4.1	13.5	484.3	6.1	3.1	26.7	4.3	9.2	3.3	2127.5
	* leaves	31.7	25.9	463.2	79.3	3.3	13.3	90.8	2.9	7.1	7.6	3.4	6.3	1.1	725.2
I29	* skin	65.3	73.7	6.2	7.5	-	-	-	-	32.2	1.0	a	a	69.6	255.5
	* flesh	23.9	-	1.7	3.1	-	-	-	-	0.5	-	-	-	2.6	31.8
	** skin	98.1	118.7	21.6	8.8	-	-	-	-	24.7	0.6	-	a	100.8	373.3
	** flesh	15.3	-	8.9	-	-	-	-	-	2.1	-	-	-	1.4	27.7
	* flowers	34.8	20.6	642.5	507.4	-	27.8	660.2	5.1	2.3	31.2	3.7	2.9	1.5	1933.4
	* leaves	40.6	29.4	422.0	510.7	-	-	273.3	3.3	9.5	26.5	5.3	1.8	1.0	1316.3
I53	* skin	37.1	49.1	31.6	5.6	6.7	-	-	-	31.4	38.2	a	a	61.6	261.3
	* flesh	19.4	-	-	2.0	5.4	-	-	-	2.5	-	a	a	-	29.3
	** skin	89.1	156.1	40.1	23.4	8.3	-	-	-	28.7	37.8	a	a	164.9	548.4
	** flesh	14.1	-	-	1.7	3.6	-	-	-	0.7	-	a	a	0.7	20.8
	* flowers	25.4	16.0	1224.8	78.1	8.3	8.7	1161.5	9.4	3.5	20.1	6.1	5.0	2.9	2558.7
	* leaves	33.9	28.4	597.4	59.9	6.3	15.1	244.8	-	7.6	12.4	10.4	2.6	-	1005.8

Table A3.2 Flavonoids continued...

Cultivar		cat	epicat	2a	2b	UF1	q-3-gal	rutin	q-3-glu	eriod	Km-3-r	My	UF2	narin	total flavonoids[x]
Kowiniwini	* skin	40.1	49.7	5.3	-	6.8	-	-	-	61.0	-	a	a	60.5	239.7
	* flesh	3.6	-	-	-	-	-	-	-	1.0	-	-	-	-	4.6
	** skin	49.4	59.6	20.4	-	5.0	-	-	-	9.7	-	a	a	66.1	219.2
	** flesh	5.8	-	5.0	-	-	-	-	-	-	-	a	-	3.2	15.6
	* flowers[1]														
	* leaves	55.9	36.0	472.3	458.9	-	16.6	253.0	3.0	17.9	19.9	1.0	1.1	0.6	1334.1
Maori Chief	* skin	62.0	72.1	9.2	-	10.5	6.9	-	-	44.8	18.3	a	a	55.2	279.0
	* flesh	7.0	-	-	-	-	-	-	-	-	-	-	-	-	7.0
	** skin	75.2	59.6	47.9	-	18.6	7.5	-	-	14.1	20.7	a	a	67.2	310.8
	** flesh	6.7	-	-	-	-	-	-	-	-	-	-	-	-	6.7
	* flowers[1]														
	* leaves	-	-	181.0	110.1	1.3	2.4	239.0	2.1	2.8	12.7	-	-	-	551.4
Moe Moe	* skin	40.4	84.9	30.2	9.0	-	-	-	-	29.1	22.2	a	a	72.0	287.8
	* flesh	5.7	-	-	-	-	-	-	-	0.8	-	-	-	1.0	7.5
	** skin	63.5	92.3	25.0	8.7	4.7	-	-	-	7.9	39.3	a	a	95.8	337.2
	** flesh	7.0	-	-	-	-	-	-	-	-	-	-	-	0.7	7.7
	* flowers	36.5	14.8	538.9	505.9	28.7	11.2	642.3	5.8	3.8	44.1	17.5	2.3	2.4	1834.4
	* leaves	28.7	16.7	317.4	460.8	-	26.0	157.5	3.6	5.6	21.7	-	-	-	1038.0

Table A3.2 Flavonoids continued...

Cultivar		cat	epicat	2a	2b	UF1	q-3-gal	rutin	q-3-glu	eriod	Km-3-r	My	UF2	narin	total flavonoids[x]
M10	* skin	50.7	21.2	-	18.6	-	-	-	-	17.9	14.9	4.9	16.8	42.1	165.4
	* flesh	4.0	-	-	-	-	-	-	-	-	-	-	-	-	4.0
	* flowers	22.4	9.7	242.1	123.6	9.3	24.3	618.3	6.4	3.3	31.8	3.5	4.5	3.1	1094.3
	* leaves	-	-	111.0	72.9	2.3	6.3	265.4	-	2.9	25.0	1.5	-	-	485.8
Ngaoutiouti	* skin	37.8	-	4.7	1.4	-	-	-	-	16.8	11.1	a	a	38.4	110.2
	* flesh	3.6	-	-	-	-	-	-	-	-	-	-	-	1.3	4.9
	** skin	83.0	-	16.9	5.9	-	-	-	-	5.7	9.2	a	a	106.6	227.3
	** flesh	7.3	-	2.4	-	-	-	-	-	-	-	-	-	1.0	10.7
	* flowers	48.4	18.0	500.6	493.4	23.7	11.3	545.4	58.3	3.9	43.1	12.7	17.9	0.7	1746.5
	* leaves	45.3	32.8	583.7	790.6	-	68.3	377.3	12.0	12.6	36.1	3.8	6.9	1.1	1959.8
Northern Star	* skin	45.0	-	11.7	2.4	4.8	-	-	-	36.2	13.0	a	a	56.5	169.6
	* flesh	-	-	-	-	-	-	-	-	1.7	-	-	-	1.0	2.7
	** skin	56.7	-	63.8	25.7	-	-	-	7.1	5.2	14.8	a	a	79.7	253.0
	** flesh	5.6	-	4.5	1.7	-	-	-	-	-	-	-	-	-	11.8
	* flowers[1]														
	* leaves	17.8	6.4	260.9	223.2	-	10.1	351.6	4.7	5.1	13.5	1.6	-	1.1	894.4

Table A3.2 Flavonoids continued...

Cultivar		cat	epicat	2a	2b	UF1	q-3-gal	rutin	q-3-glu	eriod	Km-3-r	My	UF2	narin	total flavonoids[x]
Old Red	* skin	52.3	-	14.8	4.3	7.4	8.2	-	-	35.1	52.8	a	a	59.6	234.5
	* flesh	5.1	-	-	-	-	-	-	-	-	-	-	-	-	5.1
	** skin	59.7	-	18.4	18.3	6.3	1.2	-	-	12.9	80.8	a	a	83.3	281.5
	** flesh	-	-	-	-	-	-	-	-	-	-	-	a	-	0
	* flowers[1]														
	* leaves	42.9	30.7	427.5	377.3	-	18.3	498.7	4.1	-	115.5	10.3	2.1	13.3	1423.1
O60/1	* skin	59.3	-	12.3	9.4	-	-	-	-	93.4	51.4	34.2	a	98.8	324.6
	* flesh	23.0	-	-	-	-	-	-	-	1.3	-	-	-	1.4	25.7
	** skin	107.7	-	24.1	9.5	-	3.1	-	-	13.3	a	a	a	132.7	479.6
	** flesh	11.7	-	-	-	-	-	-	-	-	-	-	-	0.5	12.2
	* flowers	49.4	9.8	23.7	24.9	9.8	10.2	1147.9	5.8	3.6	54.1	15.6	41.4	1.8	1341.0
	* leaves	61..0	21.6	16.3	15.2	4.5	-	739.4	-	16.4	44.7	6.4	7.2	8.8	927.9
Poiwa	* skin	36.8	-	16.2	4.4	-	-	-	-	20.4	13.2	a	a	67.8	158.8
	* flesh	7.2	-	-	-	-	-	-	-	-	-	-	-	-	7.2
	** skin	57.6	-	11.5	7.4	-	-	-	-	8.3	9.3	a	9.0	99.6	193.7
	** flesh	5.5	-	-	-	-	-	-	-	-	-	-	-	-	5.5
	* flowers	38.2	16.0	528.6	512.0	28.6	18.2	615.5	6.1	4.1	43.0	17.1	2.6	2.5	1812.8
	* leaves	34.2	39.7	215.3	355.0	-	4.7	114.5	4.9	-	1.9	-	-	-	770.2

Table A3.2 Flavonoids continued...

Cultivar		cat	epicat	2a	2b	UF1	q-3-gal	rutin	q-3-glu	eriod	Km-3-r	My	UF2	narin	total flavonoids[x]
Raupi	* skin	41.9	63.9	7.5	8.3	-	-	-	-	46.0	12.2	a	a	47.3	227.1
	* flesh	-	-	-	-	-	-	-	-	1.9	-	-	-	-	1.9
	** skin	65.6	83.4	24.6	7.0	-	-	-	-	4.8	22.0	a	a	46.8	254.2
	** flesh	4.0	-	4.4	-	-	-	-	-	-	-	-	-	1.1	9.5
	* flowers	33.4	13.4	513.6	807.0	44.8	11.6	783.5	-	4.0	41.0	30.3	18.3	4.1	2256.4
	* leaves	25.7	7.8	299.3	761.2	-	25.9	240.6	4.1	5.7	16.2	1.5	-	0.5	1417.0
Red Flesh	* skin	62.8	-	11.3	-	7.7	-	-	-	24.7	183.5	a	a	45.3	335.3
	* flesh	16.0	-	1.8	4.5	-	-	-	-	1.9	5.7	a	a	1.0	30.9
	** skin	147.6	-	9.6	-	31.9	-	-	-	46.5	94.0	a	a	163.3	492.9
	** flesh	20.5	-	1.6	-	-	-	-	-	1.1	-	a	a	0.8	24.0
	* flowers	29.1	12.4	371.3	303.6	-	5.6	415.1	7.0	1.8	51.2	3.2	4.2	0.6	1197.7
	* leaves	32.1	38.5	615.0	892.6	-	40.0	452.6	11.3	10.1	29.8	16.4	3.8	4.9	2126.9
Red Rascal	* skin	59.0	-	25.2	7.2	3.1	-	-	-	24.7	72.9	a	a	28.8	220.9
	* flesh	8.5	-	3.8	-	-	-	-	-	0.5	4.3	-	-	0.5	17.6
	** skin	57.0	-	23.8	10.4	2.9	-	-	-	7.9	59.7	a	-	166.0	327.7
	** flesh	9.5	-	3.8	-	-	-	-	-	-	-	-	-	1.3	16.2
	* flowers	24.3	13.0	1100.9	86.4	5.2	-	1018.1	7.9	2.7	32.2	4.5	6.5	2.0	2292.7
	* leaves	19.6	34.4	1064.4	154.2	6.3	34.2	635.3	12.8	14.2	25.4	7.1	3.0	9.5	2010.3

Table A3.2 Flavonoids continued...

Cultivar		cat	epicat	2a	2b	UF1	q-3-gal	rutin	q-3-glu	eriod	Km-3-r	My	UF2	narin	total flavonoids[x]
Red Rocks	* skin	66.1	-	14.7	-	8.0	-	-	-	45.5	29.5	a	a	52.7	216.5
	* flesh	10.2	-	-	-	-	-	-	-	-	-	-	-	-	10.2
	** skin	61.0	-	11.4	-	3.6	-	-	-	10.5	11.6	8.3	a	63.5	161.6
	** flesh	8.9	-	-	-	-	-	-	-	-	-	-	-	-	8.9
	* flowers	-	11.6	637.8	104.5	14.8	16.6	2014.2	8.4	-	44.3	3.9	6.6	2.3	2854.5
	* leaves	28.8	26.9	462.7	163.0	6.6	12.7	588.1	9.8	8.4	26.7	3.1	5.6	5.5	1339.2
Rua	* skin	24.8	-	9.7	3.1	-	-	-	-	16.3	33.4	6.7	8.7	45.9	133.2
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	41.9	-	18.0	3.5	3.1	-	-	-	5.8	27.1	-	30.5	58.3	157.7
	** flesh	4.7	-	2.1	-	-	-	-	-	-	-	-	-	-	6.9
	* flowers	24.8	16.3	418.6	223.1	-	12.9	1068.0	6.4	2.4	37.3	3.3	4.1	1.0	1810.8
	* leaves	19.2	9.9	394.2	385.1	10.1	46.1	511.7	11.1	4.6	34.9	5.5	3.9	0.7	1427.6
Russet Burbank	* skin	44.8	45.6	5.0	2.6	-	-	-	-	26.2	8.3	2.3	9.2	74.1	206.6
	* flesh	14.9	-	-	-	-	-	-	-	-	-	-	-	2.4	17.1
	** skin	79.1	48.2	2.3	3.5	-	-	-	-	4.5	13.6	9.8	7.0	95.7	246.9
	** flesh	18.2	-	-	-	-	-	-	-	-	-	-	-	-	18.2
	* flowers	17.5	9.7	39.8	38.7	12.8	17.0	2058.4	-	-	26.7	4.3	4.9	1.8	2222.4
	* leaves	-	-	84.3	111.6	3.1	2.9	468.9	5.0	1.7	7.6	3.4	6.3	1.6	683.7

Table A3.2 Flavonoids continued...

Cultivar		cat	epicat	2a	2b	UF1	q-3-gal	rutin	q-3-glu	eriod	Km-3-r	My	UF2	narin	total flavonoids[x]
Skerry Blue	* skin	43.7	84.9	17.3	-	-	9.4	-	-	70.9	25.9	a	a	67.0	319.1
	* flesh	10.5	2.6	-	3.4	-	-	-	-	1.0	-	-	a	-	17.5
	** skin	67.9	98.2	12.4	3.9	-	-	-	-	26.4	-	a	a	107.0	315.8
	** flesh	6.3	-	-	1.7	-	-	-	-	-	-	-	a	-	9.0
	* flowers[1]														
	* leaves	36.6	27.6	539.0	617.3	-	16.3	705.7	13.7	7.3	29.8	4.1	2.6	0.5	1993.8
Stage II Blue	* skin	15.3	83.6	4.1	5.3	2.3	-	-	-	36.4	66.1	a	a	39.0	252.1
	* flesh	8.9	-	-	-	-	-	-	-	1.1	-	a	a	-	10.0
	** skin	23.7	189.7	10.6	13.4	3.4	-	-	-	37.1	72.2	a	a	103.5	453.6
	** flesh	17.5	-	-	-	-	-	-	-	-	-	a	a	-	17.5
	* flowers	22.3	17.9	295.2	199.3	12.5	17.1	1207.4	-	2.6	31.9	3.5	4.2	2.0	1808.2
	* leaves	24.9	36.4	580.9	618.3	3.1	-	717.0	7.1	10.3	35.0	12.6	3.5	3.3	2036.3
Tekau	* skin	36.2	9.9	26.1	8.7	16.6	-	-	-	3.3	19.2	a	13.8	28.0	148.2
	* flesh	9.0	-	-	-	-	-	-	-	-	-	-	-	2.7	11.7
	** skin	63.6	-	10.0	6.3	4.9	-	-	-	4.4	24.9	a	7.7	80.4	194.5
	** flesh	7.5	-	-	-	-	-	-	-	-	-	-	-	1.3	8.8
	* flowers	24.2	8.1	32.9	38.6	13.3	14.7	2778.4	8.2	2.1	55.6	7.2	11.5	3.2	2979.4
	* leaves	53.3	20.8	21.3	13.2	5.1	29.4	883.1	8.1	9.7	48.8	7.0	3.5	-	1092.8

Table A3.2 Flavonoids continued...

Cultivar		cat	epicat	2a	2b	UF1	q-3-gal	rutin	q-3-glu	eriod	Km-3-r	My	UF2	narin	total flavonoids[x]
Urenika	* skin	65.5	-	9.4	-	-	-	-	-	36.0	-	a	a	35.5	146.4
	* flesh	13.4	-	4.4	4.4	-	-	-	-	16.9	-	a	a	2.2	41.3
	** skin	80.1	-	4.0	-	-	-	-	-	25.9	-	a	a	52.5	162.5
	** flesh	18.5	-	2.4	4.5	-	-	-	-	14.5	-	a	a	4.3	44.2
	* flowers	14.0	10.8	224.2	178.3	4.2	12.6	730.8	5.4	1.4	35.0	6.4	1.6	0.4	1217.1
	* leaves	-	1.2	185.9	379.7	-	13.7	97.3	1.9	2.3	10.0	6.1	2.7	1.3	693.3
Whitu	* skin	90.7	-	2.9	-	-	3.0	-	-	19.5	-	2.6	11.2	33.6	149.7
	* flesh	26.3	-	-	-	-	-	-	-	-	-	-	-	-	26.3
	* flowers[1]														
	* leaves	32.8	5.4	12.5	11.1	4.3	19.7	985.6	-	5.1	562.4	9.3	1.5	2.2	1641.1
177/3	* skin	50.7	36.9	11.6	-	-	6.1	-	-	20.2	4.5	2.7	10.6	28.3	158.3
	* flesh	9.3	-	-	-	-	-	-	-	-	-	-	-	-	9.3
	** skin	56.6	44.0	25.2	-	-	7.5	-	-	6.2	25.7	4.4	5.0	45.7	210.9
	** flesh	10.4	-	-	-	-	-	-	-	-	-	-	-	1.7	12.1
	* flowers	38.2	8.6	28.3	28.5	38.7	12.9	1671.3	-	2.5	65.7	14.5	12.0	2.0	1896.7
	* leaves	51.6	37.9	26.6	19.1	12.8	106.1	1777.0	13.4	17.4	97.4	211.9	6.4	5.3	2164.6

Table A3.2 Flavonoids continued...

Cultivar		cat	epicat	2a	2b	UF1	q-3-gal	rutin	q-3-glu	eriod	Km-3-r	My	UF2	narin	total flavonoids[x]
289/3	* skin	77.3	39.6	48.0	12.3	6.3	-	-	-	23.5	22.0	a	20.1	42.1	271.1
	* flesh	20.7	-	-	-	-	-	-	-	-	-	-	-	-	20.7
	** skin	74.5	72.5	17.7	8.3	16.6	-	-	-	4.8	30.7	a	a	68.5	293.6
	** flesh	16.2	-	-	-	-	-	1.8	-	-	-	-	-	-	18.0
	* flowers	30.1	17.9	403.7	149.5	56.4	17.6	1313.0	-	3.9	40.8	32.7	22.8	0.4	2033.3
	* leaves	31.1	-	295.8	151.1	1.3	16.4	473.6	1.9	3.8	16.3	-	-	-	991.3
1858.21	* skin	44.2	16.5	9.2	3.3	1.2	2.8	-	-	17.6	10.2	1.6	-	13.3	118.3
	* flesh	8.6	-	-	-	-	-	-	-	-	-	-	-	-	8.6
	** skin	54.5	66.6	18.7	5.8	5.9	-	-	-	3.4	9.7	6.9	-	56.6	221.2
	** flesh	16.2	-	-	-	-	-	-	-	-	-	-	-	-	16.2
	* flowers	12.3	13.9	665.3	456.4	-	13.4	1138.1	6.6	5.8	33.5	2.7	4.8	0.6	2345.9
	* leaves	32.0	-	247.6	222.4	1.7	8.3	154.0	2.0	0.7	20.6	-	1.4	-	689.3
Diploid	* skin	56.1	-	14.3	4.0	-	-	-	-	27.1	2.6	3.9	7.8	4.3	108.4
	* flesh	3.7	-	-	-	-	-	-	-	-	-	-	-	-	3.7
	* flowers	8.0	5.2	14.6	4.5	41.6	15.1	1076.1	5.6	0.7	65.2	104.5	23.5	0.6	1237.2
	* leaves	55.5	19.6	24.6	20.8	9.9	-	1031.9	-	14.5	37.0	10.8	-	1.8	1208.6

Table A3.3 Anthocyanins

Cultivar		UA1 (14.2)	UA2 (15.8)	Dp-3-r	Cy-3-r	UA3 (23.0)	Pg-3-r	Mv-3-r	anth-1	Pn-gly (25.3)	anth-3	anth-2	UA4 (28.0)	UA5 (34.2)	total anthocyanins
Arran Victory	* skin	47.4	-	-	-	-	-	21.1	1555.3	-	-	76.4	-	-	1700.2
	* flesh	1.8	-	-	-	-	-	0.8	91.5	-	-	12.8	-	-	106.9
	** skin	174.4	-	30.4	-	-	-	36.2	2028.7	-	-	117.2	-	-	2386.9
	** flesh	-	-	-	-	-	-	-	52.2	-	-	26.0	-	-	78.2
	* flowers[1]														
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Blue Derwent	* skin	97.5	-	15.0	-	-	-	21.9	2224.6	-	-	126.8	-	-	2485.8
	* flesh	-	-	-	-	-	-	-	10.8	-	-	-	-	-	10.8
	** skin	122.1	-	18.7	-	-	-	33.2	2737.2	-	-	178.7	-	-	3089.9
	** flesh	-	-	-	-	-	-	-	103.7	-	-	-	-	-	103.7
	* flowers[1]														
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Catriona	* skin	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	-	-	-	-	-	-	-	210.0	-	-	28.7	-	-	238.7
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	-	-	11.0	-	-	2.8	-	325.1	-	-	642.6	25.1	-	1006.6
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0

Table A3.3 Anthocyanins continued...

Cultivar		UA1 (14.2)	UA2 (15.8)	Dp-3-r	Cy-3-r	UA3 (23.0)	Pg-3-r	Mv-3-r	anth-1	Pn-gly (25.3)	anth-3	anth-2	UA4 (28.0)	UA5 (34.2)	total anthocyanins
Desirée	* skin	-	36.6	-	-	29.2	16.8	-	-	202.2	1119.2	-	-	21.0	1425.0
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	-	45.6	-	-	19.3	15.0	-	-	221.2	1144.3	-	-	19.4	1445.4
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	-	-	-	0.9	-	1.0	-	-	121.0	-	39.1	9.5	-	171.5
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
I29	* skin	-	9.1	-	-	1.3	1.4	-	-	9.0	173.7	-	-	-	194.5
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	-	30.0	9.9	-	7.6	6.5	-	-	18.6	361.6	-	-	14.0	448.2
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	-	-	-	-	-	-	-	-	17.3	-	-	-	-	17.3
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
I53	* skin	7.0	-	-	-	-	-	6.0	666.4	-	-	40.2	-	-	719.6
	* flesh	9.1	-	-	-	-	-	8.3	437.6	-	-	17.7	-	-	463.6
	** skin	68.0	-	-	-	-	-	25.9	3175.4	-	-	211.6	-	-	3480.9
	** flesh	9.3	-	-	-	-	-	12.2	473.9	-	-	26.9	-	-	522.3
	* flowers	3.9	-	-	-	-	-	-	4.4	-	-	-	-	-	8.3
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0

Table A3.3 Anthocyanins continued...

Cultivar		UA1 (14.2)	UA2 (15.8)	Dp-3-r	Cy-3-r	UA3 (23.0)	Pg-3-r	Mv-3-r	anth-1	Pn-gly (25.3)	anth-3	anth-2	UA4 (28.0)	UA5 (34.2)	total anthocyanins
Kowiniwini	* skin	24.6	-	18.1	-	-	-	10.9	1684.2	-	-	248.6	-	-	1986.4
	* flesh	-	-	-	-	-	-	-	8.9	-	-	-	-	-	8.9
	** skin	31.8	-	30.8	-	-	-	13.3	1682.1	-	-	258.0	-	-	2016.0
	** flesh	-	-	-	-	-	-	-	43.8	-	-	-	-	-	43.8
	* flowers[1]														
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Maori Chief	* skin	24.1	-	5.0	-	-	-	6.8	727.9	-	-	42.5	-	-	806.3
	* flesh	-	-	-	-	-	-	-	24.6	-	-	-	-	-	24.6
	** skin	79.2	-	49.6	-	-	-	25.2	462.3	-	-	39.8	-	-	656.1
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers[1]														
Moe Moe	* skin	10.6	-	-	-	-	-	4.3	423.3	-	-	386.5	-	-	824.7
	* flesh	-	-	-	-	-	-	-	8.3	-	-	-	-	-	8.3
	** skin	37.4	-	-	-	-	-	23.8	572.4	-	-	58.1	-	-	691.7
	** flesh	-	-	-	-	-	-	-	18.5	-	-	-	-	-	18.5
	* flowers	12.8	-	-	-	-	7.8	-	658.2	-	-	18.5	-	-	697.3
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0

Table A3.3 Anthocyanins continued...

Cultivar		UA1 (14.2)	UA2 (15.8)	Dp-3-r	Cy-3-r	UA3 (23.0)	Pg-3-r	Mv-3-r	anth-1	Pn-gly (25.3)	anth-3	anth-2	UA4 (28.0)	UA5 (34.2)	total anthocyanins
M10	* skin	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Ngaoutiouti	* skin	9.8	-	-	-	-	1.9	-	391.0	-	0	28.4	-	-	431.1
	* flesh	-	-	-	-	-	-	-	38.4	-	-	-	-	-	38.4
	** skin	29.2	-	-	-	-	11.0	-	951.7	-	-	91.8	-	-	1083.7
	** flesh	-	-	-	-	-	-	-	30.2	-	-	-	-	-	30.2
	* flowers	34.7	-	-	-	-	3.1	-	422.4		-	14.8	-	-	475.0
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Northern Star	* skin	-	-	6.7	-	-	-	-	2.7	-	-	6.0	-	-	15.4
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	-	-	54.3	-	-	-	-	41.7	-	-	66.5	-	-	162.5
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers[1]														
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0

Table A3.3 Anthocyanins continued...

Cultivar		UA1 (14.2)	UA2 (15.8)	Dp-3-r	Cy-3-r	UA3 (23.0)	Pg-3-r	Mv-3-r	anth-1	Pn-gly (25.3)	anth-3	anth-2	UA4 (28.0)	UA5 (34.2)	total anthocyanins
Old Red	* skin	8.0	-	71.6	-	-	-	9.8	560.0	-	-	760.0	-	-	1409.4
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	19.1	-	177.0	-	-	-	40.3	870.0	-	-	1485.0	-	-	2591.4
	** flesh	-	-	-	-	-	-	-	14.6	-	-	-	-	-	14.6
	* flowers[1]														
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
O60/1	* skin	-	11.3	8.2	-	1.0	3.5	-	-	27.8	125.5	-	42.6	-	220.0
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	-	54.6	28.5	-	6.8	-	-	-	24.1	456.0	-	202.8	-	772.8
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	16.9	-	13.5	-	-	8.5	-	-	806.3	-	452.4	106.7	-	1404.3
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Poiwa	* skin	10.2	-	-	-	-	-	1.5	356.0	-	-	28.7	-	-	396.4
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	15.0	-	-	-	-	-	-	231.3	-	-	-	-	-	246.3
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	9.5	-	-	-	-	6.5	-	652.1	-	-	20.0	-	-	688.1
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0

Table A3.3 Anthocyanins continued...

Cultivar		UA1 (14.2)	UA2 (15.8)	Dp-3-r	Cy-3-r	UA3 (23.0)	Pg-3-r	Mv-3-r	anth-1	Pn-gly (25.3)	anth-3	anth-2	UA4 (28.0)	UA5 (34.2)	total anthocyanins
Raupi	* skin	18.6	-	9.3	-	-	1.3	-	288.5	-	-	51.7	-	-	369.4
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	41.9	-	18.0	-	-	-	-	801.0	-	-	136.8	-	-	997.7
	** flesh	-	-	-	-	-	-	-	11.9	-	-	-	-	-	11.9
	* flowers	2.6	-	-	10.2	-	30.4	-	1293.8	-	-	210.7	44.3	-	1592.0
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Red Flesh	* skin	-	56.3	-	-	23.6	11.0	-	-	287.3	1552.9	-	659.3	22.5	2612.9
	* flesh	-	3.9	-	-	-	2.6	-	-	12.6	65.3	-	14.2	-	98.6
	** skin	-	161.4	-	-	29.3	26.3	-	-	382.6	1975.2	-	727.2	23.2	3325.2
	** flesh	-	5.1	-	-	-	4.0	-	-	9.7	60.1	-	35.9	-	114.8
	* flowers	8.5	-	6.0	-	-	-	-	-	34.3	-	10.4	-	-	59.2
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Red Rascal	* skin	-	8.6	-	-	5.5	3.0	-	-	108.6	473.0	-	-	6.3	605.0
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	-	15.6	-	-	11.8	-	-	-	89.2	363.4	-	243.1	5.0	1091.5
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	1.4	-	4.2	2.6	-	0.5	-	-	135.5	-	42.7	13.6	-	200.5
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0

Table A3.3 Anthocyanins continued...

Cultivar		UA1 (14.2)	UA2 (15.8)	Dp-3-r	Cy-3-r	UA3 (23.0)	Pg-3-r	Mv-3-r	anth-1	Pn-gly (25.3)	anth-3	anth-2	UA4 (28.0)	UA5 (34.2)	total anthocyanins
Red Rocks	* skin	-	35.7	-	-	2.5	2.6	-	-	21.8	156.9	-	36.9	6.5	262.9
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	-	43.4	14.5	-	-	3.0	-	-	-	96.2	-	47.5	-	204.6
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	-	-	-	-	-	-	-	-	26.5	-	4.6	-	-	31.1
	* leaves	-	-	-	-		-	-	-	-	-	-	-	-	0
Rua	* skin	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	1.9	-	3.2	-	-	-	-	-	7.2	-	2.5	-	-	14.8
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Russet Burbank	* skin	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	-	-	-	-	-	-	-	-	40.6	-	-	-	-	40.6
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0

Table A3.3 Anthocyanins continued...

Cultivar		UA1 (14.2)	UA2 (15.8)	Dp-3-r	Cy-3-r	UA3 (23.0)	Pg-3-r	Mv-3-r	anth-1	Pn-gly (25.3)	anth-3	anth-2	UA4 (28.0)	UA5 (34.2)	total anthocyanins
Skerry Blue	* skin	62.3	-	-	-	-	-	9.5	1003.2	-	-	41.2	-	-	1116.2
	* flesh	-	-	-	-	-	-	-	89.3	-	-	-	-	-	89.3
	** skin	173.3	-	23.5	-	-	-	22.3	1294.9	-	-	65.2	-	-	1579.2
	** flesh	-	-	-	-	-	-	-	33.9	-	-	-	-	-	33.9
	* flowers[1]														
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Stage II Blue	* skin	-	-	28.9	-	-	-	-	932.4	-	-	1682.1	-	-	2643.4
	* flesh	-	-	-	-	-	-	-	15.4	-	-	14.1	-	-	29.5
	** skin	-	-	107.7	-	-	-	46.4	2164.8	-	-	5127.7	-	-	7446.6
	** flesh	-	-	-	-	-	-	-	106.4	-	-	112.1	-	-	218.5
	* flowers	-	-	-	-	-	-	-	9.5	-	-	-	-	-	9.5
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Tekau	* skin	-	-	-	-	-	-	-	26.1	-	-	-	-	-	26.1
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	-	-	-	-	-	-	-	81.8	-	-	-	-	-	81.8
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	-	-	-	-	-	-	-	33.5	-	-	-	-	-	33.5
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0

Table A3.3 Anthocyanins continued...

Cultivar		UA1 (14.2)	UA2 (15.8)	Dp-3-r	Cy-3-r	UA3 (23.0)	Pg-3-r	Mv-3-r	anth-1	Pn-gly (25.3)	anth-3	anth-2	UA4 (28.0)	UA5 (34.2)	total anthocyanins
Urenika	* skin	13.3	-	96.5	-	-	-	19.5	1614.4	-	-	2647.3	-	-	4376.4
	* flesh	6.7	-	41.6	-	-	-	27.6	1174.7	-	-	1069.7	-	-	2320.3
	** skin	-	-	147.7	-	-	-	39.3	1402.8	-	-	4189.0	-	-	5778.8
	** flesh	-	-	42.1	-	-	-	18.4	445.2	-	-	845.9	-	-	1351.6
	* flowers	-	-	-	-	-	3.3	-	167.5	-	-	19.0	-	-	189.8
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Whitu	* skin	-	-	9.8	-	-	-	-	-	-	-	6.8	-	-	16.6
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers[1]														
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
177/3	* skin	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	19.4	-	4.6	-	-	9.6	-	574.5	-	-	86.2	13.1	-	707.4
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0

Table A3.3 Anthocyanins continued...

Cultivar		UA1 (14.2)	UA2 (15.8)	Dp-3-r	Cy-3-r	UA3 (23.0)	Pg-3-r	Mv-3-r	anth-1	Pn-gly (25.3)	anth-3	anth-2	UA4 (28.0)	UA5 (34.2)	total anthocyanins
289/3	* skin	-	-	-	-	-	-	-	24.3	-	-	-	-	-	24.3
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	-	-	-	-	-	-	-	263.8	-	-	43.5	-	-	307.3
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	-	-	-	-	-	23.8	-	1344.3	-	-	256.3	53.8		1678.2
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
1858.21	* skin	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	** skin	-	-	-	-	-	-	-	-	-	-	51.1	-	-	51.1
	** flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	7.3	-	9.2	-	-	-	-	80.8	-	-	13.1	1.3	-	111.7
	* leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Diploid	* skin	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	* flowers	11.9	-	-	11.1	-	-	212.6	5046.6	-	-	567.9	65.0	-	5915.1
	* leaves	-	-	-	-	-	-	-	149.6	-	-	7.6	-	-	157.2

Table A3.4 Phenolic acids in white, purple and red tubers.

	Colour	gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total	no
Skin*	White	8.2	195.5	0	847.6	75.7	48.4	92.8	3.2	3.5	7.1	5.1	33.3	9.4	7.7	2.4	1339.6	9
	Purple	10.3	288.7	0	1624.2	171.3	85.0	72.8	6.2	11.2	15.8	7.5	74.1	17.0	9.8	2.0	2438.3	14
	Red	9.0	318.2	0	1673.7	105.7	183.5	75.4	5.2	13.2	25.0	30.6	73.8	44.7	36.1	3.1	2637.7	6
Flesh*	Wfl/wsk	6.0	91.3	0	46.2	0.6	9.8	2.7	0	10.0	0.3	6.2	2.0	0	0	0.1	160.1	10
	Wfl/colsk	5.5	118.6	0	63.6	0.4	11.3	9.5	0.1	9.2	0.3	4.1	2.8	0	0.2	0	237.7	10
	Purfl/pursk	6.4	114.7	0	481.2	1.2	28.0	33.2	1.0	11.0	1.9	4.7	3.6	1.9	0	0.3	688.1	3
	Redfl/redsk	5.0	125.6	0	486.4	0	34.0	47.8	0.5	15.9	7.2	5.8	1.6	6.5	0	0.3	736.1	1
Skin**	White	6.6	223.4	0	1660.9	228.9	82.0	34.0	7.5	5.2	10.3	14.7	23.8	6.5	15.7	3.4	2327.7	8
	Purple	8.4	270.3	0	2474.5	410.2	102.1	88.5	14.1	8.3	10.0	35.3	21.8	15.0	9.7	2.7	3507.5	13
	Red	7.0	268.6	0	2138.0	308.7	110.2	136.3	7.1	7.0	22.1	49.6	40.6	34.5	32.6	3.6	3129.4	6
Flesh**	Wfl/wsk	1.3	53.0	0	46.0	8.6	10.8	2.9	0.1	0.1	1.4	3.9	0.4	0	0	0	122.6	7
	Wfl/colsk	1.0	86.0	0	105.5	3.9	10.5	8.3	0.2	0.8	2.1	4.4	0.5	0	1.3	0	219.5	10
	Purfl/pursk	2.4	81.1	0	878.7	19.3	37.8	68.4	1.5	0.3	3.8	17.1	0.7	1.6	0.6	0	734.4	3
	Redfl/redsk	1.9	75.8	0	408.2	9.7	48.3	47.5	0	0	17.2	2.1	0	4.3	13.6	0	628.6	1

Table A3.5 Flavonoids in white, purple and red tubers

	Colour	cat	epicat	2a	2b	UF1	rutin	eriod	Km-3-r	My	UF2	narin	total	no
Skin*	White	49.7	21.6	14.5	5.6	2.9	0	23.4	14.3	3.5	11.8	36.9	170.1	9
	Purple	47.5	44.3	15.1	4.3	3.8	0	40.8	25.5	a	a	56.9	240.7	14
	Red	62.6	26.2	15.4	4.0	3.4	0	37.2	75.4	a	a	57.4	281.6	6
Flesh*	Wfl/wsk	8.6	0	0	0	0	0	0.2	0	0	0	0.6	9.4	10
	Wfl/colsk	10.0	0	0.5	0.3	0.8	0	0.5	0	0	0	0.6	12.7	10
	Purfl/pursk	13.9	0	1.5	2.1	1.8	0	3.5	4.6	a	a	0.7	28.1	3
	Redfl/redsk	16.0	0	0	0	3.1	0	1.9	5.7	a	a	1.0	27.7	1
Skin**	White	67.5	41.6	20.3	8.4	3.8	0	5.7	19.8	3.8	10.0	67.8	235.8	8
	Purple	67.2	60.8	19.6	8.2	4.6	0	17.5	24.3	a	a	95.5	298.4	13
	Red	95.3	26.9	19.2	4.8	7.0	0	21.4	35.7	a	a	122.2	333.0	6
Flesh**	Wfl/wsk	10.3	0	0.8	0.2	0	0	0.7	0	0	0	0.4	12.4	7
	Wfl/colsk	7.5	0	1.9	0	0	0	0.2	0	0	0	0.9	10.5	10
	Purfl/pursk	16.7	0	0.8	2.1	0	0	5.1	0	a	a	0.7	25.4	3
	Redfl/redsk	20.5	0	1.6	0	0	0	1.1	0	a	a	0.8	24.0	1

Table A3.6 Anthocyanins in white, purple and red tubers

	Colour	UA1 (14.2)	UA2 (15.8)	Dp-3-r	Cy-3-r	UA3 (23.0)	Pg-3-r	Mv-3-r	anth-1	Pn-gly	anth-3	anth-2	UA4 (28.0)	UA5 (34.2)	total	no
Skin*	White	0	0	1.7	0	0	0	0	5.3	0	0	2.5	0	0	8.2	9
	Purple	25.6	0	18.8	0	0	0	8.8	955.9	0	0	473.9	0	0	1483.1	14
	Red	0	23.2	1.4	0	8.1	5.0	0	0	92.6	506.9	0	13.3	7.6	658.1	6
Flesh*	Wfl/wsk	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10
	Wfl/colsk	0	0	0	0	0	0	0	6.5	0	0	0	0	0	6.5	10
	Purfl/pursk	5.3	0	13.9	0	0	0	12.0	542.6	0	0	367.2	0	0	937.8	3
	Redfl/redsk	0	3.9	0	0	0	2.6	0	0	12.6	65.3	0	14.2	0	98.6	1
Skin**	White	0	0	6.8	0	0	0	0	74.7	0	0	23.7	0	0	105.2	8
	Purple	60.6	0	46.1	0	0	0	24.4	1413.4	0	0	919.9	0	0	2463.5	13
	Red	0	58.4	8.8	0	12.5	8.5	0	0	122.6	732.8	0	203.4	10.3	1214.6	6
Flesh**	Wfl/wsk	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7
	Wfl/colsk	0	0	0	0	0	0	0	9.1	0	0	0	0	0	9.1	10
	Purfl/pursk	3.1	0	14.0	0	0	0	10.2	341.8	0	0	328.3	0	0	697.5	3
	Redfl/redsk	0	5.1	0	0	0	4.0	0	0	9.7	60.1	0	35.9	0	114.8	1

APPENDIX 4

Concentration (μg/gFW) of phenolic acids, flavonoids and anthocyanins in *Solanum* spp

Key

Phenolic acids

gal	gallic acid
pro	protocatechuic acid
p-ben	*p*-hydroxybenzoic acid
chl	chlorogenic acid
caf	caffeic acid
UP1	unknown phenolic acid 1
van	vanillic acid
syr	syringic acid
coum	*p*-coumaric acid
fer	ferulic acid
UP2	unknown phenolic acid 2
sin	sinapic acid
sal	salicylic acid
UP3	unknown phenolic acid 3
cinn	cinnamic acid

Flavonoids

cat	catechin
epicat	epicatechin
2a	flavonoid 2a (quercetin-glucose-rhamnose)
2b	flavonoid 2b (quercetin-glucose-rhamnose)
UF1	unknown flavonoid 1
q-3-gal	quercetin-3-galactoside
q-3-glu	quercetin-3-glucoside
eriod	eriodictyol
Km-3-r	kaempferol-3-rutinoside
My	myricetin
UF2	unknown flavonoid 2
narin	naringenin

Anthocyanins

UA1 (14.2)	unknown anthocyanin 1 - retention time 14.2min
Cy-3-rut	cyanidin-3-rutinoside
Mv-3-rut	malvidin-3-rutinoside
anth-1	petunidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside
anth-2	malvidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside

total[x]	sum of total flavonoids excluding myricetin and UF2, as in a number of cultivars reliable concentrations of these compounds could not be obtained (see 'a'), so all totals were comparable
a	due to co-elution of myricetin and UF2 with anthocyanins, reliable concentrations of these could not be determined
-	not detected (*i.e.* concentration equals zero)

Table A4.1 Phenolic acids

Species		gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total
S. acaule	skin	12.7	52.0	-	696.1	179.2	116.4	14.2	1.8	73.0	54.2	-	8.9	20.7	1.3	6.1	1236.6
subsp. *acaule*	flesh	8.9	45.7	-	39.3	2.5	14.0	0.9	-	31.0	1.5	-	-	-	-	-	143.8
(ACL 2113)	flowers	37.7	56.2	21.0	2985.9	8.9	100.7	19.3	14.8	11.9	27.5	3.3	22.3	28.0	-	5.7	3343.1
n=3	leaves	2.7	41.2	-	1397.0	4.0	54.4	22.9	3.3	5.8	7.3	3.2	0.5	5.7	1.4	1.3	1550.5
S. acaule	skin	9.3	60.6	-	387.3	227.9	48.6	16.4	-	61.0	19.7	-	10.4	6.3	1.8	2.7	851.7
subsp. *aemulans*	flesh	8.9	105.1	-	23.7	3.2	8.9	7.3	-	98.4	-	-	-	-	-	-	255.3
(ACLAEM 3734)	flowers	58.9	154.2	21.7	3241.8	17.7	49.4	17.5	38.0	19.0	23.6	11.8	-	-	-	1.8	3655.3
n=2	leaves	-	57.6	11.0	1662.6	6.8	43.6	10.3	3.1	2.3	9.9	12.7	-	22.7	0.8	5.2	1848.3
S. berthaultii	skin	20.8	116.4	8.3	2262.0	89.1	56.3	145.1	-	18.7	16.8	-	17.5	-	-	-	2750.9
(BER 4036)	flesh	10.5	83.0	-	353.4	2.8	76.9	62.3	-	19.7	14.0	-	4.8	3.7	-	0.4	631.3
n=2	flowers	36.7	51.4	17.3	2958.2	26.1	55.0	19.6	99.4	25.3	81.1	18.0	66.6	-	-	10.1	3464.4
	leaves	181.1	116.5	24.3	1116.3	12.4	46.7	22.5	29.6	10.8	26.5	28.3	10.7	-	-	6.4	1632.1
S. gourlayi	skin	11.5	52.4	3.3	852.6	158.4	31.0	14.1	-	21.1	5.3	41.3	11.8	11.6	5.5	-	1225.1
(GRL 2480)	flesh	7.5	36.0	-	21.0	0.8	4.3	-	-	19.8	-	-	-	4.5	-	-	93.7
n=2	flowers	26.2	57.0	65.6	1645.9	8.2	40.4	19.4	40.2	10.2	23.3	14.2	29.9	115.7	9.2	8.6	2113.8
	leaves	59.1	68.2	15.5	641.7	4.3	16.2	8.8	5.4	7.0	5.7	7.7	3.4	24.1	1.7	5.7	874.1

Table A4.1 Phenolic acids continued...

Species		gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total
S. oplocense	skin	8.4	40.4	-	335.1	164.8	20.0	25.6	-	9.3	7.3	11.9	11.0	15.7	4.2	-	653.5
(OPL 3777)	flesh	4.1	44.0	-	11.5	5.8	5.5	7.7	-	12.6	1.2	-	-	6.3	-	-	98.6
n=2	flowers	27.0	651.7	60.9	731.4	3.8	18.2	3.4	16.3	9.4	8.6	4.8	24.4	57.9	13.6	9.2	1640.1
	leaves	28.2	294.3	-	52.4	-	2.1	2.7	-	0.3	1.3	-	2.2	4.6	0.5	0.6	389.0
S. sanctae-rosae	skin	11.0	42.4	-	335.0	128.7	21.5	8.0	-	17.9	12.1	-	7.1	18.6	-	-	602.1
(SCT 3269)	flesh	2.4	26.3	-	24.3	4.0	5.7	-	-	12.7	1.1	-	-	5.2	3.4	-	84.9
n=2	flowers	71.5	280.1	27.5	2540.4	-	26.3	19.3	40.7	10.6	18.4	17.3	46.3	61.1	11.2	2.3	3172.8
	leaves	-	155.2	6.4	1348.8	10.2	21.2	44.1	6.9	6.6	5.7	6.6	-	11.2	-	1.1	1623.4
S. sanctae-rosae	skin	12.8	61.9	-	439.0	176.2	35.0	6.5	2.0	34.9	27.5	-	4.3	11.8	7.5	0.4	819.8
(SCT 3779)	flesh	8.1	62.9	-	25.5	3.4	5.7	3.0	-	33.3	4.0	-	-	0.7	-	-	146.5
n=3	flowers	76.5	192.6	11.2	1335.8	10.6	29.9	29.7	13.5	18.1	14.4	9.2	20.4	35.9	-	2.7	1800.6
	leaves	-	416.7	24.1	455.5	5.6	206.8	13.7	-	3.5	5.0	3.6	2.7	28.9	4.4	2.0	1172.4
S. sparsipilum	skin	13.5	46.6	-	1389.5	375.4	50.8	12.7	1.5	20.8	23.9	49.4	12.7	103.1	9.1	0.8	2108.0
(SPL 3488)	flesh	9.3	20.9	-	45.9	3.0	8.8	2.3	-	19.1	-	-	-	5.6	-	-	114.8
n=2	flowers	23.0	28.9	27.1	2461.5	16.6	74.2	5.0	66.5	24.5	22.0	21.2	42.3	-	-	4.2	2837.6
	leaves	46.9	42.9	25.8	503.8	5.6	18.7	51.5	-	7.9	6.8	-	10.7	11.8	-	0.7	732.8

Table A4.1 Phenolic acids continued...

Species		gal	pro	p-ben	chl	caf	UP1	van	syr	coum	fer	UP2	sin	sal	UP3	cinn	total
S. sparsipilum	skin	12.8	52.0	7.6	718.6	260.9	43.5	4.2	1.6	10.8	4.6	-	9.8	-	6.0	0.3	1082.5
(SPL 3563)	flesh	7.5	99.8	1.2	57.7	3.0	10.3	3.4	-	4.8	2.7	2.5	-	4.7	-	-	248.3
n=2	flowers	44.9	98.7	34.8	4707.1	10.6	32.3	-	87.5	36.8	29.2	12.6	37.2	-	-	4.4	5135.8
	leaves	80.0	96.2	16.6	2353.3	7.1	73.6	55.9	34.7	20.9	16.8	8.1	24.3	12.9	15.2	6.9	2822.0
S. speggazzinii	skin	15.8	63.8	1.5	1080.6	125.2	45.3	20.3	-	107.3	52.2	-	6.1	10.7	5.3	8.2	1542.0
(SPG 3745)	flesh	9.1	45.3	-	142.1	2.4	16.7	1.4	-	46.5	3.9	2.8	-	4.6	-	-	274.6
n=2	flowers	30.2	42.2	19.6	2875.5	8.3	51.0	10.6	28.3	78.3	28.9	27.8	35.9	35.4	41.7	3.9	3317.3
	leaves	52.3	157.3	106.4	387.4	4.4	24.0	48.2	-	9.0	12.5	17.5	9.4	13.5	-	12.0	854.6
S. speggazzinii	skin	13.3	70.8	-	884.4	232.5	58.5	29.3	1.1	87.7	38.5	-	11.3	12.3	2.2	21.0	1462.6
(SPG 3791)	flesh	6.1	31.0	-	25.5	2.6	10.5	-	-	8.5	0.7	4.7	-	6.7	-	-	95.9
n=2	flowers	37.5	36.1	-	1537.0	24.0	39.6	25.7	62.8	45.4	32.6	10.7	17.6	23.1	20.4	1.9	1914.3
	leaves	72.4	68.4	30.0	87.2	5.2	7.8	13.4	-	4.6	8.6	9.8	58.5	16.6	-	1.6	330.9
S. stenotomum	skin	19.7	113.2	8.0	2103.2	505.8	70.3	24.7	5.5	61.0	18.4	1.9	75.0	19.9	3.0	6.5	3035.7
(STN 4711)	flesh	4.0	44.0	-	294.8	9.7	34.5	6.4	-	12.1	2.3	-	-	-	-	-	254.7
n=2	flowers	32.6	71.4	-	4102.1	15.4	86.6	52.1	31.2	21.6	20.8	8.6	13.4	59.6	19.3	6.5	4540.8
	leaves	35.5	96.6	17.5	1102.1	0.2	58.9	19.9	6.4	11.0	11.4	11.5	12.9	17.3	12.8	1.6	1415.2

Table A4.2 Flavonoids

Species		cat	epicat	2a	2b	UF1	q-3-gal	rutin	q-3-glu	eriod	Km-3-r	My	UF2	narin	total[x]
S. acaule	skin	21.4	17.1	-	9.0	-	-	-	-	33.3	-	6.1	7.7	7.0	87.8
subsp. *acaule*	flesh	4.4	4.7	-	-	-	-	-	-	-	-	-	-	-	9.1
(ACL 2113)	flowers	143.4	37.1	12.9	35.0	243.9	126.0	8867.6	-	11.3	106.7	a	-	19.8	9603.7
n=3	leaves	60.2	4.1	3.0	2.7	5.3	64.9	357.0	2.3	2.0	14.0	2.0	6.0	1.0	514.7
S. acaule	skin	17.2	10.5	-	-	-	-	-	-	17.9	-	a	-	10.4	55.9
subsp. *aemulans*	flesh	-	-	-	-	-	-	-	-	0.8	-	-	-	1.2	2.0
(ACLAEM 3734)	flowers	130.4	16.9	40.3	61.8	33.5	89.6	8204.5	-	3.6	212.1	a	-	-	8792.5
n=2	leaves	48.9	29.1	6.9	3.1	4.2	20.8	271.0	6.3	4.6	22.7	-	25.4	-	417.7
S. berthaultii	skin	60.7	27.2	3.0	5.5	-	-	-	-	40.1	1.9	1.4	-	19.6	157.8
(BER 4036)	flesh	12.2	2.8	-	-	-	-	-	-	1.7	-	-	-	6.3	22.9
n=2	flowers	231.4	21.2	37.7	75.4	69.4	22.6	6536.9	6.2	68.8	397.9	a	15.2	15.0	7482.3
	leaves	383.7	15.2	2.0	-	4.5	28.4	607.3	14.9	19.2	65.2	-	17.1	1.7	1141.9
S. gourlayi	skin	22.5	-	-	1.9	-	-	-	-	5.5	-	-	-	16.2	46.0
(GRL 2480)	flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	-
n=2	flowers	86.7	45.9	-	16.8	278.7	30.1	5851.7	-	9.2	292.7	a	17.1	14.5	6626.3
	leaves	34.1	10.8	-	7.6	4.4	19.0	214.8	6.4	1.7	15.5	a	13.3	1.2	315.4

Table A4.2 Flavonoids continued...

Species		cat	epicat	2a	2b	UF1	q-3-gal	rutin	q-3-glu	eriod	Km-3-r	My	UF2	narin	total[x]
S. oplocense	skin	9.3	2.8	-	-	-	-	-	-	4.2	-	-	-	7.0	23.3
(OPL 3777)	flesh	-	4.7	-	-	-	-	-	-	-	-	-	-	-	4.7
n=2	flowers	32.9	19.5	35.0	18.2	141.6	51.8	5804.2	-	13.6	827.9	a	a	7.0	6956.4
	leaves	37.6	8.2	-	4.9	-	10.5	80.4	0.6	0.5	17.7	1.8	7.5	-	140.4
S. sanctae-rosae	skin	14.2	-	-	-	-	-	-	-	15.5	-	-	-	9.5	38.7
(SCT 3269)	flesh	7.7	-	-	-	-	-	-	-	1.5	-	-	-	-	9.2
n=2	flowers	32.3	14.1	2.8	14.3	177.1	58.0	6073.4	39.3	51.1	406.7	a	a	24.0	6892.9
	leaves	46.8	5.8	-	1.0	1.0	24.5	276.8	24.0	22.4	23.5	a	15.0	-	425.6
S. sanctae-rosae	skin	17.4	11.9	-	-	-	-	-	-	25.4	-	a	-	16.5	71.2
(SCT 3779)	flesh	1.7	-	-	-	-	-	-	-	-	-	-	-	-	1.7
n=3	flowers	129.2	34.7	11.2	16.3	47.0	94.9	3783.7	5.4	60.6	121.0	a	54.5	9.1	4313.1
	leaves	51.9	28.8	3.9	1.4	2.9	7.5	111.8	20.8	14.1	13.0	1.9	102.4	-	256.3
S. sparsipilum	skin	16.1	6.5	-	0.4	-	-	-	-	13.5	5.9	a	-	54.3	96.7
(SPL 3488)	flesh	-	-	-	-	-	-	-	-	-	-	-	-	-	-
n=2	flowers	29.0	11.3	1478.8	247.2	212.0	16.7	1768.9	-	84.0	82.0	a	21.0	6.7	3932.2
	leaves	40.8	16.3	156.8	22.3	3.2	40.6	160.6	2.9	9.9	9.3	5.0	17.0	1.8	464.2

Table A4.2 Flavonoids continued...

Species		cat	epicat	2a	2b	UF1	q-3-gal	rutin	q-3-glu	eriod	Km-3-r	My	UF2	narin	total[x]
S. sparsipilum	skin	33.4	9.2	-	-	-	-	-	3.1	28.1	31.0	a	12.2	30.0	134.7
(SPL 3563)	flesh	4.1	-	-	-	-	-	-	-	0.5	-	-	-	-	4.6
n=2	flowers	143.5	6.2	24.5	16.0	446.3	24.5	5460.8	-	133.1	246.6	a	21.1	11.9	6513.2
	leaves	150.4	29.1	8.2	3.9	9.6	160.7	719.1	-	13.0	44.5	a	44.9	8.9	1147.1
S. speggazzinii	skin	13.3	7.5	-	20.8	-	-	-	-	5.3	3.2	a	-	23.5	73.4
(SPG 3745)	flesh	-	2.4	-	13.0	-	-	-	-	-	-	-	-	-	15.3
n=2	flowers	44.8	29.0	1602.5	116.3	22.8	24.4	1026.6	80.5	41.7	24.4	a	28.4	2.5	3015.3
	leaves	51.2	22.5	6.1	26.8	12.4	27.0	13.6	19.7	-	13.8	6.4	20.9	-	186.4
S. speggazzinii	skin	35.6	-	-	12.3	-	-	-	-	27.0	3.2	a	-	28.1	106.1
(SPG 3791)	flesh	-	-	-	-	-	-	-	-	2.3	-	-	-	-	2.3
n=2	flowers	105.0	28.4	2555.0	173.8	30.1	18.5	927.4	20.3	26.0	27.7	a	6.1	2.7	3914.6
	leaves	74.9	13.7	20.7	6.4	5.6	4.5	10.7	13.6	10.2	10.8	2.7	7.4	-	170.9
S. stenotomum	skin	74.8	10.0	-	10.1	2.9	13.7	-	-	3.0	10.8	5.5	2.2	45.7	170.9
(STN 4711)	flesh	19.8	-	-	2.6	-	-	-	-	-	-	-	2.1	1.4	23.8
n=2	flowers	44.0	43.9	1028.3	60.2	183.2	14.6	4309.2	12.3	19.3	86.7	a	a	2.4	5803.9
	leaves	68.6	11.7	126.2	18.6	0.9	24.9	81.7	-	12.8	10.2	5.2	10.1	1.5	325.2

Table A4.3 Anthocyanins

Species		UA1 (14.2)	Cy-3-rut	Mv-3-rut	anth-1	anth-2	total
S. acaule subsp. *acaule*	skin	-	-	-	-	-	-
(ACL 2113)	flesh	-	-	-	-	-	-
n=3	flowers	-	3.8	14.3	644.7	-	662.8
	leaves	0.5	-	-	-	-	0.5
S. acaule subsp. *aemulans*	skin	-	-	-	16.8	-	16.8
(ACLAEM 3734)	flesh	-	-	-	-	-	-
n=2	flowers	-	-	13.6	116.7	-	130.3
	leaves	12.6	-	-	-	-	12.6
S. berthaultii	skin	-	-	-	10.2	-	10.2
(BER 4036)	flesh	-	-	-	-	-	-
n=2	flowers	-	-	-	667.1	-	667.1
	leaves	-	-	-	-	-	-
S. gourlayi	skin	-	-	-	43.1	-	43.1
(GRL 2480)	flesh	-	-	-	-	-	-
n=2	flowers	21.2	4.1	23.4	1141.3	-	1189.9
	leaves	8.8	-	-	36.8	-	45.6

Table A4.3 Anthocyanins continued...

Species		UA1 (14.2)	Cy-3-rut	Mv-3-rut	anth-1	anth-2	total
S. oplocense	skin	-	-	-	-	-	-
(OPL 3777)	flesh	-	-	-	-	-	-
n=2	flowers	10.4	3.2	12.8	738.3	14.6	251.8
	leaves	0.4	-	-	0.4	0.1	0.9
S. sanctae-rosae	skin	-	-	-	-	-	-
(SCT 3269)	flesh	-	-	-	-	-	-
n=2	flowers	40.2	7.4	28.7	2254.4	90.1	2420.7
	leaves	0.7	-	0.3	9.5	-	10.5
S. sanctae-rosae	skin	-	-	13.4	260.0	-	273.4
(SCT 3779)	flesh	-	-	-	-	-	-
n=3	flowers	-	-	8.8	211.6	-	220.4
	leaves	0.6	-	0.1	2.4	0.2	3.3
S. sparsipilum	skin	-	-	-	45.2	-	45.2
(SPL 3488)	flesh	-	-	-	-	-	-
n=2	flowers	13.5	4.1	97.8	817.5	-	932.9
	leaves	8.7	-	0.2	4.9	-	13.8

Table A4.3 Anthocyanins continued...

Species		UA1 (14.2)	Cy-3-rut	Mv-3-rut	anth-1	anth-2	total
S. sparsipilum	skin	-	-	-	55.7	-	55.7
(SPL 3563)	flesh	-	-	-	-	-	-
n=2	flowers	-	8.3	192.0	1322.6	-	1522.9
	leaves	36.6	-	3.0	11.5	-	51.5
S. speggazzinii	skin	-	-	-	197.5	-	197.5
(SPG 3745)	flesh	-	-	-	-	-	-
n=2	flowers	11.1	-	-	78.8	-	89.8
	leaves	0.3	-	-	2.3	0.5	6.1
S. speggazzinii	skin	-	-	-	111.2	-	111.2
(SPG 3791)	flesh	-	-	-	-	-	-
n=2	flowers	-	-	-	89.9	-	89.9
	leaves	-	-	-	-	-	-
S. stenotomum	skin	-	-	-	-	-	-
(STN 4711)	flesh	-	-	-	-	-	-
n=2	flowers	5.2	13.4	12.9	194.5	14.6	240.6
	leaves	-	-	-	-	-	-

APPENDIX 5

Papers published in relation to thesis

Lewis, C.E., Walker, J.R.L., Lancaster, J.E., 1995. Effect of polysaccharides on the colour of anthocyanins. *Food Chemistry*, **54**: 315-319.

ELSEVIER

Food Chemistry 54 (1995) 315–319

0308-8146/95/$9.50
0308-8146(95)00026-7

Analytical, Nutritional and Clinical Methods Section

Effect of polysaccharides on the colour of anthocyanins

Christine E. Lewis, John R. L. Walker*

Dept of Plant & Microbial Sciences, University of Canterbury, Christchurch, New Zealand

&

Jane E. Lancaster

New Zealand Institute for Crop & Food Research Ltd, Private Bag 4704, Christchurch, New Zealand

(Received 4 November 1994; accepted 8 December 1994)

The effect of a variety of plant polysaccharides and sugars on anthocyanin colour was investigated. The colour intensity (absorbance), but not the λ_{max}, of solutions of different anthocyanins was found to be diminished in the presence of amylose, amylopectin and α- and β-cyclodextrins whilst glucose, maltose and sucrose caused an increase in colour. This colour change was more apparent at pH 4 than at pH 2.

INTRODUCTION

During our work with coloured potato tubers it was noticed that extracts of anthocyanins often showed decreased colour. This decrease was in contrast to flower (camellia, delphinium, mallow) extracts and it was thought that the high levels of starch present in tubers might be responsible for this effect.

Anthocyanins

Anthocyanins are found in the vacuoles of many plants and their perceived colour depends on several factors such as concentration, solvent, temperature, pH, substitution on the B-ring and presence of copigments (Brouillard, 1982). pH has a large effect on the colour of anthocyanins because the three species of water (i.e. H^+, OH^- and H_2O) are highly reactive towards anthocyanins. Thus, the solvent water plays an important role in influencing both the stability and reactivity, as well as spectral properties of the various structures adopted by anthocyanins in aqueous solutions.

In acidic aqueous solutions, four species of anthocyanin molecule may exist in equilibrium: the quinonoidal base (A), the flavylium cation (AH^+), the pseudobase or carbinol (PB), and the chalcone (C) (Brouillard, 1982; Chen & Hrazdina, 1982), thus

$$AH^+ \Leftrightarrow A + H^+ \text{ acid/base equilibrium} \quad (1)$$

$$AH^+ + H_20 \Leftrightarrow PB + H^+ \text{ hydration equilibrium} \quad (2)$$

$$PB \Leftrightarrow C \text{ ring/chain tautomeric equilibrium} \quad (3)$$

Interconversion between these structures may take place as shown in Fig. 1.

In most higher plants the pH of a mature vacuole is acidic, and a survey of many flowers has shown that the pH of the vacuole ranges from 2·5 to 7·5 (Stewart *et al.*, 1975). For highly acidic cells pigmentation is probably due to the flavylium form (AH^+) alone, whereas in the range pH 3–4, both the flavylium cation and the neutral tautomer (A) may contribute to colour, whilst at pH 4–6 the neutral tautomer dominates due to deprotonation of the hydroxylated flavylium cation (eqn (1), Fig. 1) (Brouillard, 1982). Thus pH is a major factor in anthocyanin colour (Brouillard, 1982; Chen & Hrazdina, 1982).

Protection of the flavylium ring against attack by water is absolutely necessary to maintain intensely coloured solutions. One way of retaining anthocyanin colour is by removal of water and displacement of the hydration/dehydration equilibrium towards the coloured species (i.e. reduce the extent of the hydration reaction) (Brouillard, 1983).

Copigmentation may also protect anthocyanins against hydration thus preserving their red colour. Copigments include flavonoids, polyphenols, alkaloids, amino acids and organic acids. This effect occurs only in aqueous systems and is sensitive to pH, temperature and composition of the solution (Chandra *et al.*, 1993).

*To whom correspondence should be addressed.

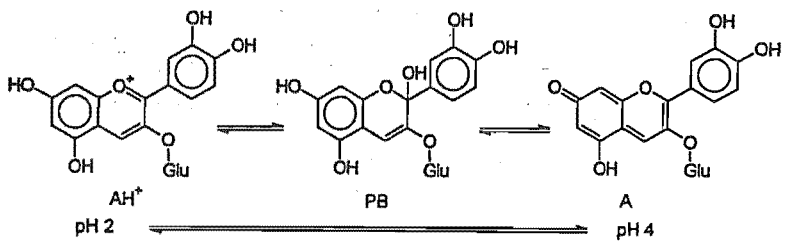


Fig. 1. Effect of pH upon the structure of cyanidin (after Brouillard, 1982). Only reactions at acid pH (i, ii) are considered here.

Plant carbohydrates

Relevant properties of selected plant and food carbohydrates are summarised below.

Most plant starches comprise 20–25% amylose and 75–80% amylopectin. Amylose consists of essentially linear chains of α-(1,4)-linked glucose with occasional α-(1,6) branch points (Morrison & Karkalas, 1990). Because amylose exists as a helical structure, with six successive glucose units per revolution and a 5Å wide cavity in the centre of the helix, it can form inclusion complexes with alcohols, fatty acids and iodine (Noltemeyer & Saenger, 1980). Amylopectin consists of α-(1,4)-glucan chains joined at numerous α-(1,6) branch points which disrupt helix formation and therefore only the short linear chains are capable of forming helices (Kennedy & White, 1979).

Cyclodextrins (CDs) are cyclic oligosaccharides containing α-(1,4)-linked glucose units; the most common are α- and β-cyclodextrin which have six and seven glucose units, respectively (Saenger, 1980; Chandra *et al.*, 1993). Cyclodextrins have hydrophobic cavities with an inner diameter of 6Å for α-CD and 7·5Å for β-CD. Thus they can form reversible inclusion complexes with smaller molecules (often phenolic substances) which fit into the cavity (in both the solid and liquid state), and are known to bind readily to molecules regardless of solvent environment. This is in marked contrast to copigmentation effects which only occur in aqueous systems (Chandra *et al.*, 1993).

Pectins are important components of plant tissues, especially of the parenchyma of fleshy roots (Coultate, 1989); for example, potato tubers contain 0·36% pectin (Robinson, 1987). Pectin is a mainly linear polymer composed of esterified galacturonic acid residues, linked by α-(1,4) glycosidic bonds (Belitz & Grosch, 1987; Robinson, 1987).

Polydextrose is composed almost entirely of randomly cross-linked glucose polymers with the α-(1,6) bond predominating (Leibrand *et al.*, 1985).

Because anthocyanin pigments in plant foods (and derived products) may occur with a variety of saccharides, the latter may affect their structure and stability thus playing a role in the final colour of food products. It was therefore of interest to investigate the effect of selected saccharides on the spectral properties of anthocyanins.

MATERIALS AND METHODS

Preparation of anthocyanins

Anthocyanin extracts were prepared from suitable plant materials:

- pelargonidin-3-glucoside (Pg-3-glu) from freeze-dried strawberries (*Fragaria ananassa*);
- delphinidin-3,5-diglucoside (Dp-3,5-glu) and delphinium-3-glucoside (Dp-3-glu) from *Delphinium* spp. petals;
- malvidin-3,5-diglucoside (Mv-3,5-glu) from mallow petals (*Malva sylvestris*);
- pelargonidin-3-*p*-coumaroyl-rutinoside-5-glucoside (Pg-RF) from tubers of potato (*Solanum tuberosum*) cultivar 'Red Flesh'; and
- malvidin-3-*p*-coumaroyl-rutinoside-5-glucoside (Mv-U) from potato cultivar 'Urenika'.

Tissue was extracted with 15% acetic acid in methanol and the anthocyanins chromatographed on a column of C_{18} silica, eluted with 60% (v/v) methanol, and redissolved in water. Each plant extract also contained other flavonoids and phenolic acids but these did not appear to have any obvious effect on the results since similar patterns were found with the pure anthocyanins. The purity of the two anthocyanin standards, cyanidin-3-rutinoside (Cy-3-rut) and malvidin-3-glucoside (Mv-3-glu) (Plantech), was confirmed by HPLC.

The pH of the anthocyanin solutions was adjusted (with HCl or NaOH) to pH 2 and/or pH 4, giving a final absorbance (at λ_{max}) of 0·6–1·0 at pH 2, or 0·4–0·8 at pH 4. The anthocyanins used are listed in Table 1.

Table 1. Sources of anthocyanins

Anthocyanin	Source	λ_{max} (nm)	
		pH 2	pH 4
Cy-3-rut	Standard (Plantech)	512	518
Dp-3,5-glu	Delphinium flowers	534/6	568
Dp-3-glu	Delphinium flowers	536/8	570
Mv-3-glu	Standard (Plantech)	518	526
Mv-3,5-glu	Mallow flowers	540	546
Pg-3-glu	Strawberry fruit	498	498
Pg-RF	Potato tubers, cv. 'Red Flesh'	504	518
Mv-U	Potato tubers, cv. 'Urenika'	524	538

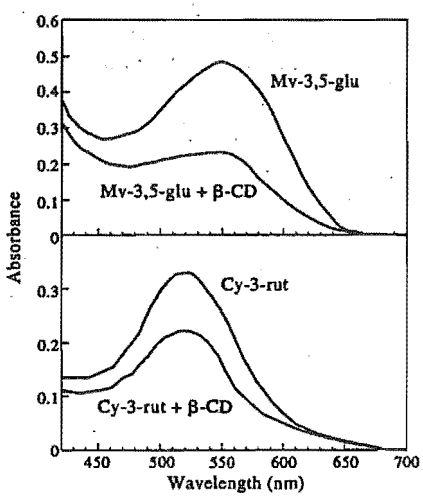


Fig. 2. Spectra of malvidin-3,5-diglucoside and cyanidin-3-rutinoside at pH 4·0 in presence or absence of β-cyclodextrin.

Carbohydrates

Solutions of the following carbohydrates were prepared by dissolving in water followed by adjustment to pH 2 or 4: 1·5% amylopectin (AP), 3% α-cyclodextrin (α-CD) (cyclohexa-amylose), 3% β-cyclodextrin (β-CD) (cyclohepta-amylose), 1·5% pectin, 3% polydextrose (PolyD, Pfizer, USA), 50% sucrose, 50% glucose, 50% fructose, and 50% maltose (all as w/v). Solutions of 1·5% amylose (AM) were dissolved initially in 50% (w/v) KOH and then the pH was adjusted (the final concentration of KOH was 12·5%). All reagents except PolyD, were supplied by Sigma (USA).

Spectrophotometric assay

For the spectrophotometric assays, equal amounts (250 μl) of anthocyanin and carbohydrate solutions were mixed and the spectra (blanked against carbohydrate solution) measured after 60 min. Controls (without added carbohydrate) contained 250 μl anthocyanin plus 250 μl distilled water (at pH 2 or 4) (or KOH/HCl blank for amylose). Results are presented as a percentage of the control absorbtion at its visible λ_{max}. Spectra were recorded on a HP8452A diode-array spectrophotometer.

RESULTS

Representative spectra showing cyanidin-3-rutinoside (standard) and mallow extract (mainly malvidin-3,5-glucoside) both without (control) and with β-cyclodextrin are given in Fig. 2 which shows the decrease in absorbance at 60 min after the addition of β-cyclodextrin. Figures 3(a)–(c) show the effect of added AM, AP,

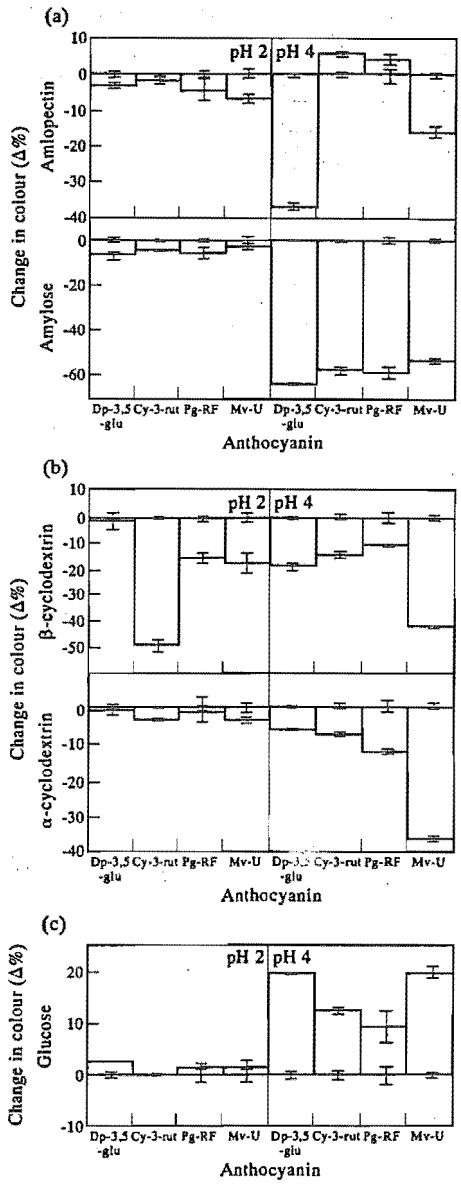


Fig. 3. Effect of added carbohydrate on colour of anthocyanins at pH 2·0 or 4·0. Results are presented as % change in absorbance at λ_{max}.

α-CD, β-CD and glucose on a selection of anthocyanins at pH 2 and 4. In the presence of AM, AP, α-CD and β-CD there was a significant decrease in visible absorbance at pH 4. However, addition of AP and α-CD showed no significant change at pH 2 whereas AM and β-CD also showed a significant decrease in absorbance at pH 2. Typically, the decrease in colour was larger at pH 4 than at pH 2. The addition of glucose resulted in an increase in absorbance, especially at pH 4. The results for sucrose and maltose are similar to the results presented in Fig. 3(c) for glucose.

Table 2. Effect of added carbohydrates on anthocyanin colour (compared with control)

Carbohydrate	ΔA at pH 2	ΔA at pH 4
Amylose[a]	Decreased	Decreased
Amylopectin[a]	No change	Decreased
α-CD[a]	No change	Decreased
β-CD[a]	Decreased	Decreased
PolyD	No change	No change
Pectin	No change	Decreased (Dp), increased (Pg and Mv)
Sucrose	No change	Increased
Glucose[a]	No change	Increased
Fructose	No change	No change
Maltose	No change	Increased
DMSO (33%)	No change	Decreased
KOH/HCl	No change	Increased

[a]See Figs 3(a)–(c) for more detail.

Results with all carbohydrates are summarised in Table 2. Polydextrose and fructose showed no significant effect at either pH, whilst pectin showed a change only at pH 4. Essentially similar results were obtained with anthocyanins from different sources. The degree of colour change appeared to be related primarily to the identity of the anthocyanin aglycone and only secondarily to its sugar substitution and/or acylation pattern.

In all cases there was no change in λ_{max}, only a change in the intensity of absorbance. The fading effect was dependent on both the anthocyanin and carbohydrate concentration. We also confirmed the observation of Yamada *et al.* (1980), that the loss of colour was time-dependent and was stable after 60 min.

DISCUSSION

From Figs 3(a)–(c) it may be seen that the addition of AM, AP, α-CD and β-CD resulted in significant decreases in anthocyanin colour with AM and β-CD showing the largest effect. The α-CD molecule has a smaller inner diameter than β-CD and thus appeared to be less efficient at forming inclusion complexes with anthocyanins. AP is a much more highly branched molecule than AM and can only form helical structures within the unbranched straight chains and therefore caused far less decrease in anthocyanin colour than did AM.

This 'fading' effect has also been reported by Yamada *et al.* (1980) who examined the interaction of only three anthocyanins (pelargonidin-3-glucoside, cyanidin-3-glucoside and delphinidin-3-(4-(*p*-coumaroyl)-L-rhamnosyl-(1,6)-glucosido)-5-glucoside) with α- and β-cyclodextrin. In their experiments each anthocyanin was adjusted to pH 2 and added to varying concentrations of α- or β-CD. Addition of β-CD resulted in the fading of the two anthocyanin solutions (Pg-3-glu and Cy-3-glu) and this effect was increased with higher concentrations of β-CD. Addition of α-CD resulted in a fading effect only for pelargonidin-3-glucoside and this was less than that measured for β-CD. This effect was found to be reversed at extremely low pH values (pH < 0·5). No change in λ_{max} was observed. Yamada *et al.* (1980) also investigated the effect of amylose (dissolved in 33% DMSO) on these anthocyanins, and found that the addition of AM only caused the fading of pelargonidin-3-glucoside, but the extent of this effect could not be determined because of immediate AM precipitation. The precipitation of AM from DMSO solutions was also observed in our experiments; it was overcome by initially dissolving AM in 50% KOH and then readjusting its pH to 2 or 4. This procedure resulted in the AM remaining in solution and retaining its helical structure, as shown by its characteristic reaction with KI/I_2 solution. Yamada *et al.* (1980) postulated that the fading phenomenon was due to the conversion of a flavylium ion into a pseudobase in two steps: (1), the formation of an inclusion complex of the anthocyanin with the CD, and (2), conversion of the flavylium ion to the pseudobase by catalytic action of the CD.

More recently Chandra *et al.* (1993) reported the stabilising effect of 0·8–1·6% α- and β-cyclodextrin on anthocyanins extracted from tart cherries with β-CD having the largest effect. Juice samples containing these cyclodextrins retained higher levels of anthocyanins after 12 weeks of storage than solutions without added cyclodextrins. This stabilisation was thought to occur because the anthocyanin was protected from attack (by water, etc.) by being in the inclusion complex. If this is so then starch (amylose) might also be expected to exhibit a similar protective effect, which could be useful in the long-term storage of foods containing anthocyanins.

The absorbance of anthocyanins (compared with the control) increased when glucose, sucrose and maltose were added, but this may be due to a decrease in water activity (a_w). Anthocyanin colour is known to increase upon the removal of water by displacement of the hydration/dehydration equilibrium (Fig. 1) towards the coloured species (Brouillard, 1983); it has been found also that sugar molecules are effective at binding water (Coultate, 1989). Copigmentation, where the aromatic residues of the copigments stack with the pyrylium ring of the flavylium cation, also reduces the extent of the hydration reaction and therefore increases the stability of the coloured species (Brouillard, 1983). Typically, copigmentation causes a bathochromic shift in the visible λ_{max} of all anthocyanins, as well as the increase in absorbtion. However, in our experiments no change in λ_{max} was observed.

The presence of sugars (and their breakdown products, furfural and 5-hydroxymethyl-furfural) in anthocyanin solutions has been found by some workers to cause anthocyanin degradation (reviewed in Francis, 1989). When sugars were added to pelagonidin-3-glucoside, fructose led to greater pigment degradation than glucose, sucrose or maltose (Francis, 1989) whilst others (R. E. Wrolstad, pers. comm.) have found an increase in stability of anthocyanins when stored in the presence of added sugars.

The effect of pectin was inconsistent; with malvidin and pelargonidin-glycosides it caused a small increase

in colour, whereas with delphinidin-3,5-glucoside it led to a small decrease in colour. Asen *et al.* (1972) have reported that pectin may act as a copigment.

The effect of carbohydrates on anthocyanin colour was more dramatic at pH 4 than at pH 2, and this was due in part to the greatly reduced absorbance of the anthocyanins at pH 4. Since the pH of plant vacuoles lies between 2·5 and 7·5 (Stewart *et al.*, 1975), it is possible that the presence of other molecules in the vacuole may have an effect on the colour shown by the anthocyanins. Similarly, in fruit juices and homogenates, the colour of anthocyanins could be affected by the levels of starch and sugars, if present, as well as the pH and other copigments. This phenomenon could be of importance for industries which rely on the natural colour of anthocyanins to produce food products with pleasing appearances.

ACKNOWLEDGEMENTS

The authors wish to thank the following: NZ Institute for Crop & Food Research Ltd, for growing the potatoes and providing material support, and the University of Canterbury for provision of research facilities.

REFERENCES

Asen, S., Stewart, R. N. & Norris, K. H. (1972). Co-pigmentation of anthocyanins in plant tissues and its effect of color. *Phytochemistry*, **11**, 1139–44.

Belitz, H. D. & Grosch, W. (1987). *Food Chemistry*. Springer-Verlag, Berlin, Germany.

Brouillard, R. (1982). Chemical structure of anthocyanins. In *Anthocyanins as Food Colors*, ed. P. Markakis. Academic Press, New York, USA, pp. 1–40.

Brouillard, R. (1983). The *in vivo* expression of anthocyanin colour in plants. *Phytochemistry*, **22**, 1311–65.

Chandra, A., Nair, M. G. & Iezzoni, A. F. (1993). Isolation and stabilization of anthocyanins from tart cherries (*Prunus cerasus* L.). *J. Agric. Food Chem.*, **41**, 1062.

Chen, L. J. & Hrazdina, G. (1982). Structural transformation reactions of anthocyanins. *Experientia*, **38**, 1030–2.

Coultate, T. P. (1989). *Food: The Chemistry of its Components* (2nd edn). Royal Society of Chemistry, London, UK.

Francis, F. J. (1989). Food colorants: anthocyanins. *Crit. Rev. Food Sci. Nutr.*, **28**, 273–314.

Kennedy, J. F. & White, C. A. (1979). Polysaccharides. In: *Comprehensive Organic Chemistry. The Synthesis and Reactions of Organic Compounds* (Vol. 5. Biological Compounds), ed. E. Haslan. Pergamon Press, Oxford, UK, pp. 755–815.

Leibrand, J. T., Smiles, R. E. & Freeman, T. M. (1985). Functions and applications for polydextrose in foods. *Food Technol. Aust.*, **37**, 166–7.

Morrison, W. R. & Karkalas, J. (1990). Starch. In *Methods in Plant Biochemistry 2: Carbohydrates*, ed. P. M. Dey. Academic Press, London, UK, pp. 323–52.

Noltemeyer, M. & Saenger, W. (1980). Structural chemistry of linear α-cyclodextrin-polyiodide complexes: X-ray crystal structures of (α-cyclodextrin)$_2$.LiI$_3$.I$_2$.8H$_2$O and (α-cyclodextrin)$_2$.Cd$_{0.5}$.I$_5$.27H$_2$O. Models for the blue amylose-iodine complex. *J. Am. Chem. Soc.*, **102**, 2710–22.

Robinson, D. S. (1987). *Food: Biochemistry and Nutritional Value*. Longman Scientific and Technical, Harlow, UK.

Saenger, W. (1980). Cyclodextrin inclusion compounds in research and industry. *Angew. Chem. Int. Ed. Engl.*, **19**, 344–62.

Stewart, R. N., Norris, K. H. & Asen, S. (1975). Microspectrophotometric measurement of pH and pH effect on color of petal epidermal cells. *Phytochemistry*, **14**, 937–42.

Yamada, T., Komiya, T. & Akaki, M. (1980). Formation of an inclusion complex of anthocyanin with cyclodextrin. *Agric. Biol. Chem.*, **44**, 1411–13.